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Isolation of Human Monoclonal Antibodies That Potently Neutralize Human Cytomegalovirus Infection by Targeting Different Epitopes on the gH/gL/UL128-131A Complex▽

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Human cytomegalovirus (HCMV) is a widely circulating pathogen that causes severe disease in immunocompromised patients and infected fetuses. By immortalizing memory B cells from HCMV-immune donors, we isolated a panel of human monoclonal antibodies that neutralized at extremely low concentrations (90% inhibitory concentration [IC_{90}] values ranging from 5 to 200 pM) HCMV infection of endothelial, epithelial, and myeloid cells. With the single exception of an antibody that bound to a conserved epitope in the UL128 gene product, all other antibodies bound to conformational epitopes that required expression of two or more proteins of the gH/gL/UL128-131A complex. Antibodies against gB, gH, or gM/gN were also isolated and, albeit less potent, were able to neutralize infection of both endothelial-epithelial cells and fibroblasts. This study describes unusually potent neutralizing antibodies against HCMV that might be used for passive immunotherapy and identifies, through the use of such antibodies, novel antigenic targets in HCMV for the design of immunogens capable of eliciting previously unknown neutralizing antibody responses.

Human cytomegalovirus (HCMV) is a member of the herpesvirus family which is widely distributed in the human population and can cause severe disease in immunocompromised patients and upon infection of the fetus. HCMV infection causes clinical disease in 75% of patients in the first year after transplantation (58), while primary maternal infection is a major cause of congenital birth defects including hearing loss and mental retardation (5, 33, 45). Because of the danger posed by this virus, development of an effective vaccine is considered of highest priority (51).

HCMV infection requires initial interaction with the cell surface through binding to heparan sulfate proteoglycans (8) and possibly other surface receptors (12, 23, 64, 65). The virus displays a broad host cell range (24, 53), being able to infect several cell types such as endothelial cells, epithelial cells (including retinal cells), smooth muscle cells, fibroblasts, leukocytes, and dendritic cells (21, 37, 44, 54). Endothelial cell tropism has been regarded as a potential virulence factor that might influence the clinical course of infection (16, 55), whereas infection of leukocytes has been considered a mechanism of viral spread (17, 43, 44). Extensive propagation of HCMV laboratory strains in fibroblasts results in deletions or mutations of genes in the UL131A-128 locus (1, 18, 21, 36, 62, 63), which are associated with the loss of the ability to infect endothelial cells, epithelial cells, and leukocytes (15, 43, 55, 61). Consistent with this notion, mouse monoclonal antibodies (MAbs) to UL128 or UL130 block infection of epithelial and endothelial cells but not of fibroblasts (63). Recently, it has been shown that UL128, UL130, and UL131A assemble with gH and gL to form a five-protein complex (thereafter designated gH/gL/UL128-131A) that is an alternative to the previously described gCIII complex made of gH, gL, and gO (22, 28, 48, 63).

In immunocompetent individuals T-cell and antibody responses efficiently control HCMV infection and reduce pathological consequences of maternal-fetal transmission (13, 67), although this is usually not sufficient to eradicate the virus. Albeit with controversial results, HCMV immunoglobulins (Igs) have been administered to transplant patients in association with immunosuppressive treatments for prophylaxis of HCMV disease (56, 57), and a recent report suggests that they may be effective in controlling congenital infection and preventing disease in newborns (32). These products are plasma derivatives with relatively low potency *in vitro* (46) and have to be administered by intravenous infusion at very high doses in order to deliver sufficient amounts of neutralizing antibodies (4, 9, 32, 56, 57, 66).

The whole spectrum of antigens targeted by HCMV-neutralizing antibodies remains poorly characterized. Using specific immunoabsorption to recombinant antigens and neutralization assays using fibroblasts as model target cells, it was estimated that 40 to 70% of the serum neutralizing activity is directed against gB (6). Other studies described human neutralizing antibodies specific for gB, gH, or gM/gN viral glycoproteins (6, 14, 26, 29, 34, 41, 52, 60). Remarkably, we have recently shown that human sera exhibit a more-than-100-fold-higher potency in neutralizing infection of endothelial cells than infection of fibroblasts (20). Similarly, CMV hyperimmunoglobulins have on average 48-fold-higher neutralizing activities against epithelial cell entry than against fibroblast entry (10). However, epitopes that are targeted by the antibodies

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that comprise epithelial or endothelial cell-specific neutralizing activity of human immune sera remain unknown.

In this study we report the isolation of a large panel of human monoclonal antibodies with extraordinarily high potency in neutralizing HCMV infection of endothelial and epithelial cells and myeloid cells. With the exception of a single antibody that recognized a conserved epitope of UL128, all other antibodies recognized conformational epitopes that required expression of two or more proteins of the gH/gL/UL128-131A complex.

MATERIALS AND METHODS

Isolation of human monoclonal antibodies and microneutralization assays. Following written consent, blood samples were collected from a heart transplant recipient with reactivated infection (donor A) and three pregnant women with HCMV primary infection (donors B, C, and D) 5 to 12 months after onset of infection. The study was approved by the Bioethical Committee of the Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. Memory B cells were isolated from peripheral blood mononuclear cells (PBMC) by positive selection with CD22 magnetic beads (Miltenyi Biotec), followed by removal of IgA^+, IgM^+, and IgD^+ cells by cell sorting. The resulting IgG^+ memory B cells were immortalized with Epstein-Barr virus (EBV) in the presence of R848 according to a previously described method (59) and plated at 10 cells/well in 384-well plates. After 2 to 3 weeks, culture supernatants were screened in parallel in microneutralization assays on fibroblasts (MRC-9; European Collection of Animal Cell Cultures) and retinal epithelial cells (ARPE-19; ATCC). Supernatants were mixed with an equal volume of HCMV clinical isolate VR1814 (42) and incubated for 2 h at room temperature before addition to target cell monolayers in 96-well plates. After 36 to 72 h, cells were fixed, permeabilized, and stained with a murine monoclonal anti-pp72 antibody (19) followed by incubation with anti-mouse antibody conjugated to AF488 (Molecular Probes). Cultures were inspected by fluorescence microscopy with automatic acquisition of 9 images/well on a BD Pathway instrument (BD Biosciences) with AttoVision software. Cells from positive cultures were cloned by limiting dilution at 0.5 cells/well. Uniqueness of the antibodies was confirmed by determination of the sequences of the variable genes. Antibodies were purified from culture supernatants by affinity chromatography on HiTrap protein A or protein G columns (GE Healthcare) followed by desalting on a HiTrap desalting column (GE Healthcare) and biotinylation with the EZ-Link NHS-PEO solid-phase biotinylation kit (Pierce).

Potency of antibodies was determined by mixing serial dilutions of the antibodies with a fixed amount of virus, giving 150 to 500 infected cells/field of analysis. The number of infected cells was plotted against the concentration of the antibody. Nonlinear regression analysis with Prism 4 GraphPad software was applied to obtain sigmoidal dose-response curves (variable slope), and inhibitory concentrations resulting in 50% or 90% inhibition of infection (IC_{50} or IC_{90}, respectively) were calculated. Target cells were human fibroblasts (MRC-9), retinal epithelial cells (ARPE-19), human umbilical vein endothelial cells (HUVEC; Lonza), monocyte-derived dendritic cells (Mo-DC) (49), and human bone marrow mesenchymal stromal cells (BM-MSC) (39). In some experiments clinical isolates VR6952, VR3480B1, and VR4760 (3, 42, 50) were used on HUVEC.

Cloning and expression of HCMV genes. Intronless, full-length UL128 (HindIII and XbaI), UL130 (HindIII and XbaI), UL131A (HindIII and XbaI), gH (HindIII and XbaI), gL (HindIII and XbaI), gB (HindIII and XbaI), gM (HindIII and XbaI, with C-terminal His tag), and gN (HindIII and XbaI, with C-terminal FLAG tag) were cloned in pcDNA3 vector (Invitrogen) by PCR with *Pfu* turbo (Stratagene) on cDNA of VR1814-infected MRC-9 cells, using primers introducing the desired restriction sites. Constructs were transfected in HEK293T cells (ATCC) with Fugene HD (Roche). Cells were harvested 24 to 48 h after transfection and fixed with 2% formaldehyde. Staining was performed with antibodies diluted in 0.5% saponin in phosphate-buffered saline (PBS) with 1% fetal bovine serum (FBS), followed by $F(ab')_2$ fragment goat anti-human IgG, which was Fcγ fragment specific and conjugated to Cy5 (Jackson ImmunoResearch). Samples were acquired on a FACSCalibur or with an automated BD FACS Array instrument (BD Biosciences) and analyzed with FlowJo software. Ectopic expression of antigens was confirmed by intracellular staining with mouse MAbs anti-UL128 and UL130 (kindly provided by T. Shenk [63]), gH (clone 5E259; US Biological), gB (clone 2F12; GeneTex), anti-FLAG (Sigma), or anti-His (Santa Cruz Biotechnology) or by Western blotting on lysates with rabbit serum anti-UL131A (kindly provided by B. Adler [1]).

UL128 pan-mutant. Amino acid sequences of UL128 from different clinical isolates (2) were aligned, and a sequence including mutations at all sites described was designed (MNSKDLTPFLTTLWLLLDHSRVPRVRAEECCEVIN VNHPPERCYDFKMCNLFTVALRCPDGEVCYSPEKTAEIRGIVTTMTH SLTRQVIHNKLTSCNYNPLYLEADGRIRCGKVSDKAQYLLGAAGSVPY RWINLEYDKITRIVGLDQYLESVKKHKRLDVCRAKMGYMLQ). The artificial gene encoding this UL128 pan-mutant was synthesized (Genart), subcloned into pcDNA3.1(+) (Invitrogen), and transfected in HEK293T cells. Cells expressing UL128 pan-mutant were costained with 15D8 and mouse anti-UL128 (63), followed by $F(ab')_2$ fragment goat anti-human IgG, which was Fcγ fragment specific and conjugated to Cy5 (Jackson ImmunoResearch), and $F(ab')_2$ fragment goat anti-mouse Ig conjugated to phycoerythrin (PE; Dako).

RESULTS

Isolation of a panel of HCMV-neutralizing human monoclonal antibodies. IgG^+ memory B cells were isolated from PBMC of HCMV-seropositive patients (three pregnant women with primary HCMV infection and one heart transplant recipient with reactivated HCMV infection) and immortalized with EBV according to a previously described method (59). In order to isolate a representative panel of HCMV-neutralizing antibodies, the primary screening was a neutralization assay that used an HCMV clinical isolate (VR1814) and either human fibroblasts (MRC-9) or epithelial cells (ARPE-19) as target cells. Based on potency of neutralization and cell target specificity, selected cultures were cloned by limiting dilution in order to isolate B-cell clones secreting neutralizing monoclonal antibodies.

Twenty-seven independent B-cell clones were isolated and characterized (Table 1 and Fig. 1). Of these, 10 produced antibodies that neutralized HCMV infection of fibroblasts as well as of epithelial cells (group 1 antibodies). These antibodies showed a spectrum of potencies with IC_{90} values ranging from 0.1 μg/ml to 30 μg/ml (~0.7 to 200 nM) and were able to neutralize infection of primary cells such as HUVEC, Mo-DC, and BM-MSC (Table 2 and data not shown). A second group of 17 B-cell clones produced antibodies that neutralized infection of epithelial cells with extraordinarily high potency (group 2 antibodies), with IC_{90} values ranging from 0.0007 μg/ml to 0.03 μg/ml (~5 to 200 pM) (Table 1 and Fig. 1). These antibodies did not neutralize infection of fibroblasts but potently neutralized infection of HUVEC and Mo-DC with IC_{50} values ranging from 0.00024 to 0.019 μg/ml (Table 2).

Taken together, the above results delineate two broad groups of HCMV-neutralizing antibodies with distinct specificities. A first group of antibodies have a broad cell target specificity since they neutralize infection of all target cells tested in the nanomolar range. A second group of antibodies does not inhibit infection of fibroblasts but neutralizes with an extraordinarily high potency (in the picomolar range) infection of epithelial, endothelial, and myeloid cells.

Recognition of the gH/gL/UL128-UL131A complex by HCMV-neutralizing antibodies. Consistent with recognition of structural viral antigens and not host cell determinants, the isolated antibodies stained punctate structures in the perinuclear region of fibroblasts at late stages of infection (not shown). Given the essential role of UL128, UL130, and UL131A gene products in HCMV endothelial cell tropism, we hypothesized that group 2 antibodies, which neutralized infection of epithelial and endothelial cells but not of fibroblasts, would bind to the product of one of these genes. Staining of MRC-9 cells that

TABLE 1. HCMV-neutralizing antibodies isolated from immune donors[a]

Group[b] and MAb	Donor	Ig isotype	IC_{90} (μg/ml) for cell line:	
			MRC-9	ARPE-19
1				
3G16	A	IgG3, λ	1	0.3
11B12	D	IgG1, κ	3.5	1.2
13H11	B	IgG1, κ	1.12	0.4
2B11	A	IgG3, λ	0.75	0.2
4H9	A	IgG3, λ	10	0.4
5F1	A	IgG3, λ	0.5	0.1
6B4	A	IgG1, κ	1	0.15
7H3	A	IgG1, λ	3	0.6
10C6	A	IgG3, λ	0.75	0.2
6L3	A	IgG1, κ	30	10
2				
15D8	B	IgG3, λ	NN[c]	0.008
1F11	A	IgG1, λ	NN	0.001
2F4	A	IgG1, λ	NN	0.001
4I22	A	IgG1, λ	NN	0.0015
4N10	C	IgG1, κ	NN	0.02
5A2	B	IgG3, κ	NN	0.023
8L13	A	IgG1, λ	NN	0.001
10F7	D	IgG1, λ	NN	0.002
10P3	A	IgG3, λ	NN	0.0025
2C12	D	IgG1, λ	NN	0.006
6G4	A	IgG1, κ	NN	0.0045
7B13	D	IgG1, λ	NN	0.003
7I13	D	IgG1, κ	NN	0.008
8J16	D	IgG1, κ	NN	0.0008
8C15	D	IgG1, λ	NN	0.0025
9I6	A	IgG1, κ	NN	0.0007
8I21	A	IgG1, κ	NN	0.03

[a] HCMV VR1814 was incubated with different dilutions of the indicated human MAbs for 2 h, and the mixture was added to monolayers of fibroblasts (MRC-9) or epithelial cells (ARPE-19). Infected cells were detected by staining with a mouse anti-pp72 MAb after 48 h. The IC_{90} values correspond to the MAb concentration that leads to 90% reduction of infected cells.

[b] Group 1, MAbs neutralizing infection of fibroblasts and epithelial cells; group 2, MAbs neutralizing infection of epithelial cells but not fibroblasts.

[c] NN, nonneutralizing at the highest concentration tested (10 μg/ml).

had been infected with either VR1814 or a mutant virus carrying a deletion of the UL132-128 region (RVFIXΔUL132-128) (21) indicated that 1F11, 2F4, and 6G4 (but not group 1 antibody 5F1) indeed recognize a product that requires an intact UL132-128 gene locus (data not shown).

To map antibody specificities, HCMV genes UL128, UL130, and UL131A were overexpressed in HEK293T cells and transfected cells were stained with the monoclonal antibodies. Surprisingly, with the exception of antibody 15D8, which stained UL128-transfected cells, all the other antibodies did not stain single-gene transfectants (Fig. 2 and data not shown). We therefore considered the possibility that the antibodies might recognize epitopes that require coexpression of more than one gene product. Indeed, eight group 2 antibodies (1F11, 2F4, 4I22, 4N10, 5A2, 8L13, 10F7, and 10P3) stained cells coexpressing UL130 and UL131A, while seven group 2 antibodies (2C12, 6G4, 7B13, 7I13, 8C15, 8J16, and 9I6) stained cells coexpressing UL128, UL130, and UL131A, and finally one antibody (8I21) stained cells transfected with UL128 and UL130 as well as with gH and gL. All these antibodies also stained HEK293T cells transfected with all genes forming the gH/gL/UL128-131A complex (Fig. 2 and data not shown).

These results demonstrate that human monoclonal antibodies that potently neutralize infection of epithelial, endothelial, and myeloid cells bind to the gH/gL/UL128-131A complex. With the exception of antibody 15D8, which binds to UL128, all antibodies bind epitopes that require coexpression of two or more HCMV proteins.

Antigenic sites in the gH/gL/UL128-131A complex recognized by group 2 neutralizing antibodies. To map the antigenic sites of the gH/gL/UL128-131A complex, we performed cross-competition experiments. HEK293T cells transfected with the five HCMV genes were preincubated with a 20-fold excess of unlabeled competitor antibodies followed by addition of a biotin-labeled antibody. By this means seven distinct antigenic sites could be identified (Table 3). Site 1 is present in UL128 and is defined by antibody 15D8. Sites 2 to 4 are formed by the combination of UL130 and UL131A and are defined by the antibodies 1F11, 2F4, 4I22, and 10F7 (site 2); 4N10 and 5A2 (site 3); and 10P3 (site 4), respectively. Sites 5 and 6 are formed by the combination of UL128, UL130, and UL131A and are defined by antibodies 2C12, 6G4, 8C15, 8J16, and 9I6 (site 5) and 7I13 (site 6), respectively. Finally, site 7 is formed by the combination of gH, gL, UL128, and UL130 and is defined by antibody 8I21. Antibodies defining site 7 and site 3 partially competed with each other (Table 3), suggesting that these sites may be close in the structure of the gH/gL/UL128-131A complex.

The above results delineate seven distinct antigenic sites in the gH/gL/UL128-131A complex and suggest that there might be a hierarchy of immunodominance among these sites, with UL130/131A being a major target of neutralizing antibodies. Interestingly, when we performed a primary screening of B-cell culture supernatants using staining of HEK293T cells transfected with UL130/131A, all binding antibodies were found to be also neutralizing. In contrast, when the same screening strategy was performed with gB or gH transfectants, several binding antibodies that lacked neutralizing activity were isolated (not shown).

Taken together, these findings indicate that neutralizing antibodies produced in humans during the course of a natural infection can be used as tools to identify relevant antigenic regions in a complex human pathogen such as HCMV.

Specificity of group 1 antibodies that neutralize infection of epithelial cells and fibroblasts. Next we determined the antigenic specificity of group 1 antibodies that neutralized infection of all target cells tested, including fibroblasts. Staining of HEK293T-transfected cells revealed that 3G16, 11B12, and 13H11 bind to gH, whereas 2B11, 4H9, 5F1, 6B4, 7H3, and 10C6 bind to gB. One antibody, 6L3, stained cells cotransfected with gM and gN. Cross-competition experiments indicated the presence of at least three distinct neutralizing antigenic sites on gB and two on gH (Tables 4 and 5 and data not shown). Antibody 6B4 reacted by enzyme-linked immunosorbent assay (ELISA) with gB 69-78 peptide (50% effective concentration [EC_{50}] of 0.044 μg/ml) (data not shown), which was previously defined as a target of human neutralizing antibodies (30, 31). Of note, gH-specific antibodies did not compete with antibody 8I21, which binds to gH/gL/UL128/130, nor with any

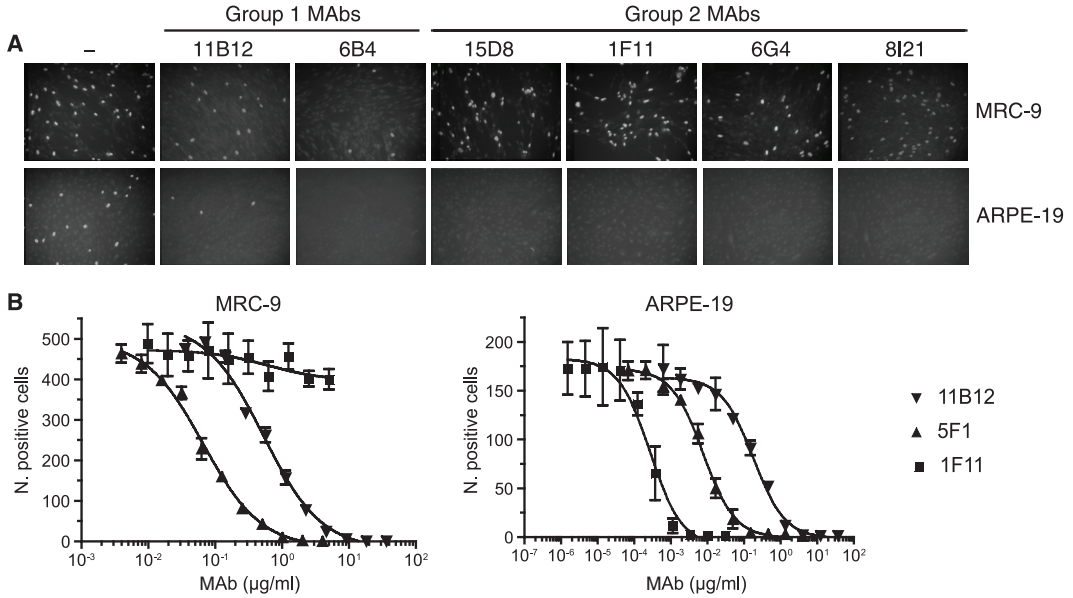

FIG. 1. Microneutralization assays on human fibroblasts and epithelial cells reveal two types of HCMV human monoclonal neutralizing antibodies. Human IgG memory B cells from HCMV-immune donors were immortalized with EBV and B-cell clones producing MAbs capable of neutralizing HCMV infection of fibroblasts (MRC-9) or retinal epithelial cells (ARPE-19) were isolated. (A) An appropriate dilution of the HCMV clinical isolate VR1814 was incubated for 2 h in the absence or presence of MAbs at 1 μg/ml before addition to target cells. Infected cells were detected by staining with anti-pp72 mouse MAb 48 h after infection. Representative staining of antibodies belonging to the two groups is shown (one field of acquisition of nine fields analyzed). (B) Microneutralization assays with different concentrations of three representative antibodies performed as for panel A. The mean of measurements of duplicate wells ± standard error from one experiment out of three performed was plotted, and nonlinear sigmoidal dose-response fit curves for each MAb were calculated. IC_{90} values for all antibodies and target cells are shown in Table 1.

of the other antibodies directed against the gH/gL/UL128-131A complex (data not shown).

In summary, targeting any of the glycoprotein complexes of the envelope is sufficient to completely block infection of endothelial and epithelial cells, indicating that besides UL128/UL130/UL131A other proteins are also essential for infection of such cell types.

Breadth of neutralizing activity of group 1 and group 2 human monoclonal antibodies. In contrast to other HCMV genes, UL128, UL130, and UL131A genes are relatively conserved among HCMV isolates and may therefore represent an attractive target for vaccination (2). We therefore investigated whether antibodies neutralizing the VR1814 isolate might be capable of neutralizing different HCMV strains. The three antibodies tested (1F11, 2F4, and 6G4) neutralized to the same extent infection of HUVEC by the reference virus VR1814 and by the three additional isolates VR6952, VR3480B1 (ganciclovir resistant), and VR4760 (ganciclovir and foscarnet resistant) (Table 2) (3, 42, 50).

TABLE 2. Neutralization of HCMV clinical isolates infecting primary cells[a]

Group and MAb	IC_{50} (μg/ml) for cell type and clinical isolate					
	HUVEC				Mo-DC (VR1814)	MSC (VR1814)
	VR1814	VR6952[b]	VR3480B1[c]	VR4760[c]		
1						
5F1	0.21	0.21	0.21	0.21	0.05	0.3
6B4	ND[d]	ND	ND	ND	0.11	2
7H3	ND	ND	ND	ND	0.06	2
10C6	0.19	0.19	0.19	0.19	0.02	0.3
2						
1F11	0.0034	0.0034	0.0034	0.0034	0.0003	>17
2F4	0.0033	0.0033	0.0033	0.0033	0.00024	>12
6G4	0.019	0.019	0.019	0.019	0.00024	>8.5

[a] Neutralization assays were performed as for Table 1 using HCMV clinical isolates and primary cells.
[b] From urine of a congenitally infected baby.
[c] From blood of an AIDS patient.
[d] ND, not done.

UL128 is the most conserved gene of the UL132-128 locus, and we found that it is a target of antibodies with potent neutralizing activity, such as 15D8. However, sequences derived from several clinical isolates revealed the existence of 10 variants with one or more mutations compared to the VR1814 sequence (2). We therefore investigated whether the binding of 15D8 would be affected by any of these mutations. Published amino acid sequences of variants of UL128 from clinical isolates (2) and laboratory strains (Towne, TB40/E, AD169, Merlin, and Toledo) were aligned, and a gene encoding a protein that includes all amino acid substitutions described as well as an additional mutation that we found to be generated at very high frequency in vitro upon PCR amplification (F33V) was synthesized. HEK293T cells were transfected with the original UL128 gene from VR1814 or with the pan-mutated gene and stained with serial dilutions of 15D8. As shown in Fig. 3, the original and the pan-mutated UL128 protein were recognized by 15D8 with comparable efficiencies (saturated staining at ~0.2

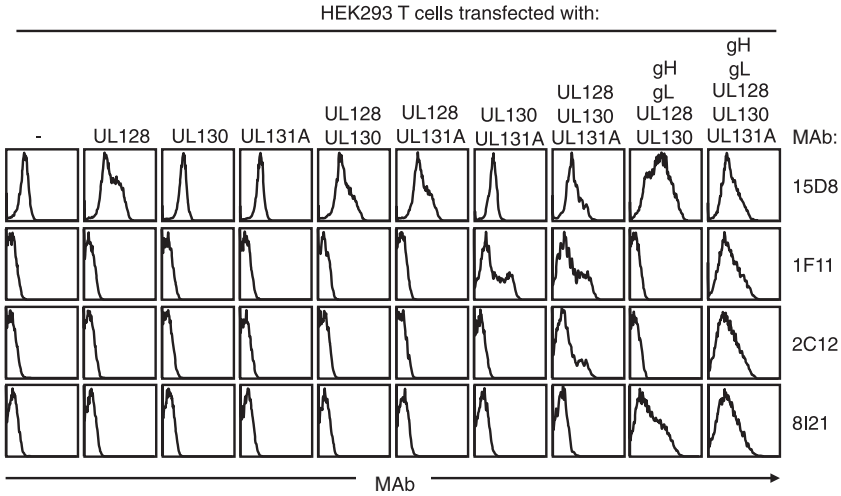

FIG. 2. Staining of HEK293T cells expressing HCMV antigens determines the antigenic specificity of human MAbs. HCMV genes UL128, UL130, UL131A, gH, and gL were cloned and transfected in HEK293T cells in different combinations. The cells were fixed, permeabilized, and stained with the neutralizing antibodies. Ectopic expression of antigens was confirmed by reactivity with mouse antibodies (not shown). Shown are representative profiles for MAbs 15D8, 1F11, 2C12, and 8I21.

μg/ml [data not shown]). These findings indicate that 15D8 recognizes a highly conserved epitope in the UL128-encoded protein.

DISCUSSION

By combining an efficient method of memory B-cell immortalization with a screening for neutralization of HCMV infection of different target cells, we have been able to isolate a panel of HCMV-neutralizing human antibodies representative of those elicited during natural infection. The antibodies fall into two main groups. A first group includes 10 antibodies that bind to previously identified targets, including gH, gB, and gM/gN. These antibodies neutralize in the nanomolar range infection of all cell types tested, including fibroblasts. In contrast, a second group includes 17 antibodies that bind to several novel epitopes of the gH/gL/UL128-131A complex. They neutralize at picomolar concentrations infection of epithelial, endothelial, and myeloid cells but not infection of fibroblasts. These findings illustrate the advantages of interrogating the human immune response to a human pathogen using viral neutralization as a primary screening.

Genetic and biochemical studies demonstrated the existence

TABLE 3. Antigenic sites on the gH/gL/UL128-131A complex recognized by group 2 human HCMV-neutralizing antibodies

Unlabeled competitor[a]	MAb specificity	Inhibition of binding (%) of biotinylated MAbs to cells expressing the gH/gL/UL128-131A complex[b]													Assigned antigenic site[c]
		15D8	1F11	2F4	4I22	10F7	4N10	5A2	2C12	6G4	8C15	9I6	8J16	8I21	
15D8	UL128	100	0	0	0	0	0	0	0	0	ND[d]	ND	ND	0	1
1F11	UL130/131A	0	100	100	100	100	0	0	0	0	ND	ND	ND	0	2
2F4	UL130/131A	ND	100	100	100	100	0	0	0	0	ND	0	ND	0	2
4I22	UL130/131A	ND	100	100	100	100	0	0	0	ND	ND	ND	0	0	2
8L13	UL130/131A	ND	100	ND	ND	100	ND	ND	ND	ND	ND	ND	ND	ND	2
10F7	UL130/131A	0	100	100	100	100	0	0	0	0	ND	ND	ND	0	2
4N10	UL130/131A	0	0	0	0	0	100	100	0	0	ND	ND	ND	90	3
5A2	UL130/131A	ND	0	50	0	0	100	100	0	0	ND	0	ND	92	3
10P3	UL130/131A	0	0	0	0	ND	0	ND	0	0	ND	ND	ND	0	4
2C12	UL128/130/131A	0	0	0	0	0	0	0	100	100	100	100	100	0	5
6G4	UL128/130/131A	0	0	0	0	0	0	0	100	100	100	100	100	0	5
8C15	UL128/130/131A	ND	ND	ND	0	ND	ND	ND	100	100	100	100	100	0	5
9I6	UL128/130/131A	ND	0	0	0	ND	ND	ND	100	100	100	100	100	0	5
7B13	UL128/130/131A	ND	ND	ND	ND	ND	ND	ND	100	100	100	100	100	0	5
8J16	UL128/130/131A	ND	0	0	0	ND	ND	ND	100	100	100	70	100	0	5
7I13	UL128/130/131A	ND	0	ND	ND	ND	ND	ND	0	0	0	0	0	0	6
8I21	gH/gL/UL128/130	0	0	0	0	ND	90	95	0	0	ND	0	ND	100	7

[a] HEK293T cells transfected with gH/gL/UL128-131A genes were permeabilized and incubated with a 20-fold excess of unlabeled competitor MAb followed by staining with biotinylated MAbs. Antibodies 10P3 and 7I13 were tested at 100-fold excess. Specificity of the MAb was assigned based on the minimum requirements for positive staining of HEK293T cells transfected with HCMV genes in different combinations (not shown).

[b] Percent inhibition for each antibody combination was calculated as follows: 100 − [(% transfected cells stained in the presence of competitor/% transfected cells in the absence of competitor) × 100].

[c] Antigenic sites are assigned on the basis of cross-competition profiles.

[d] ND, not done.

TABLE 4. Antigenic sites of group 1 human HCMV-neutralizing antibodies specific for gH[a]

Unlabeled competitor	Inhibition of binding (%) of biotinylated MAb[b]:			Assigned antigenic site[c]
	3G16	11B12	13H11	
3G16	100	15	14	1
11B12	6	100	100	2
13H11	0	100	100	2

[a] HEK293T cells transfected with the gH gene were permeabilized and incubated with a 20-fold excess of unlabeled competitor MAb followed by staining with biotinylated MAbs. The specificity of the MAbs indicates their binding to gH-expressing cells.
[b] Percent inhibition for each antibody combination was calculated as follows: 100 − [(% transfected cells stained in the presence of competitor/% transfected cells in the absence of competitor) × 100].
[c] Antigenic sites are assigned on the basis of cross-competition profiles.

TABLE 5. Antigenic sites of group 1 human HCMV-neutralizing antibodies specific for gB[a]

Unlabeled competitor	Inhibition of binding (%) of biotinylated MAb[b]:						Assigned antigenic site[c]
	2B11	4H9	5F1	10C6	6B4	7H3	
6B4	0	5	0	0	100	6	1
7H3	0	0	0	0	0	100	2
2B11	100	100	100	100	0	0	3
4H9	100	100	100	100	0	0	3
5F1	100	100	100	100	0	0	3
10C6	100	100	100	100	0	3	3

[a] HEK293T cells transfected with the gB gene were permeabilized and incubated with a 20-fold excess of unlabeled competitor MAb followed by staining with biotinylated MAbs. The specificity of the MAbs indicates their binding to gB-expressing cells.
[b] Percent inhibition for each antibody combination was calculated as follows: 100 − [(% transfected cells stained in the presence of competitor/% transfected cells in the absence of competitor) × 100].
[c] Antigenic sites are assigned on the basis of cross-competition profiles.

of a molecular complex formed by gH, gL, and products of the UL131A-128 locus which is required for HCMV infection of endothelial, epithelial, and myeloid cells (1, 18, 21, 36, 62, 63). This complex was expected to be the target of neutralizing antibodies, but attempts to produce neutralizing antibodies by immunizing animals with single proteins of the complex have met with only limited success. Here we report for the first time that neutralizing antibodies that bind to UL128, UL130, and UL131A are elicited during natural infection. Additionally, we show that such antibodies have an extraordinarily high potency in neutralizing HCMV infection in vitro. For instance, antibodies 1F11, 2F4, and 6G4 show IC_{50} values on primary cells (HUVEC and dendritic cells) as low as 0.0003 μg/ml (~2 pM). Recent studies have shown that HCMV-seropositive donors have titers of antibodies that neutralize infection of endothelial and epithelial cells that are much higher than the titers of antibodies neutralizing infection of fibroblasts (10, 20). In light of our findings, such high titers appear to be due to the potency rather than the amount of antibodies directed against the gH/gL/UL128-131A complex. Although we have no explanation for the extraordinary potency of these antibodies, we can speculate that this may be due to a combination of high affinity and high accessibility to target epitopes on infectious viral particles. The complexity of the antigens that bind such antibodies did not permit us to make affinity measurements. According to the occupancy model, viral neutralization is mediated by steric hindrance of the virus surface by antibody coating (35). In line with this notion, a possible explanation of the significant difference in potency between the two groups of antibodies might come from the relative abundance of the target antigens. It is possible that the gH/gL/UL128-131A complex is present exclusively on infectious virus, while other glycoproteins such as gB or gH may be present also on defective viral particles or on cellular debris. Antibodies specific for such target antigens could therefore bind to nonfunctional proteins and not be available for neutralization (38).

Another peculiar feature of these monoclonal antibodies is that they recognize antigenic sites that require coexpression of two or more HCMV genes. It remains to be established whether the antibodies recognize a quaternary structure made by juxtaposed proteins or a conformational epitope within one protein that requires pairing with additional proteins in order to be properly displayed. Using cross-competition experiments, we were able to assign each antibody to one of seven distinct sites, thus providing a preliminary antigenic map of the gH/gL/UL128-131A complex (Fig. 4). Notably, several antibodies isolated from different donors recognized three antigenic sites formed by the combination of UL130 and UL131A, suggesting that epitopes within these two proteins are immunodominant. It is interesting that all the antibodies isolated against the UL128/UL130/UL131A complex display neutralizing activity and that we were not able to isolate from immune donors antibodies that would bind this complex but fail to neutralize. These findings suggest that the proteins encoded by the UL131A-128 locus are presented to the immune system as tight complexes (rather than as single proteins) that are capable of eliciting protective immunity (47, 63). This may also explain the poor neutralizing capacity of antibodies raised by immunizing animals with single recombinant UL128, UL130, or UL131A protein (1, 20, 63).

It is generally assumed that infection of epithelial cells contributes to interhost transmission and viral spread, while infection of fibroblasts and smooth muscle cells allows efficient viral replication. In this context, the interplay between endothelial cells and leukocytes represents the pathogenetic basis for all clinical syndromes originating during disseminated HCMV infection (37, 43, 53). In line with the notion that cell-to-cell spreading requires UL131A-128 gene products (25), preliminary experiments indicate that, similarly to mouse antibodies to UL128 or UL130 (20), antibodies 1F11 and 2F4 block transfer of HCMV from HUVEC or human embryonic lung fibroblasts to leukocytes (not shown). Therefore, the ability to neutralize viral entry into endothelial and epithelial cells is expected to be critical for protection from viral pathogenicity.

Sequence variation among different strains is a common issue in vaccine development against viruses, including HCMV, for which unusually high variation of a subset of genes has been described elsewhere (11). Interestingly, in contrast to genes such as gO that show only 45% identical amino acid sequences in isolates from different sources (40), the UL131A-128 gene locus appears to be more conserved, with 91% amino acid identity of the gene products (2). We have been able to demonstrate that antibody 15D8 is directed against a highly conserved epitope within UL128 and that antibodies 1F11, 2F4,

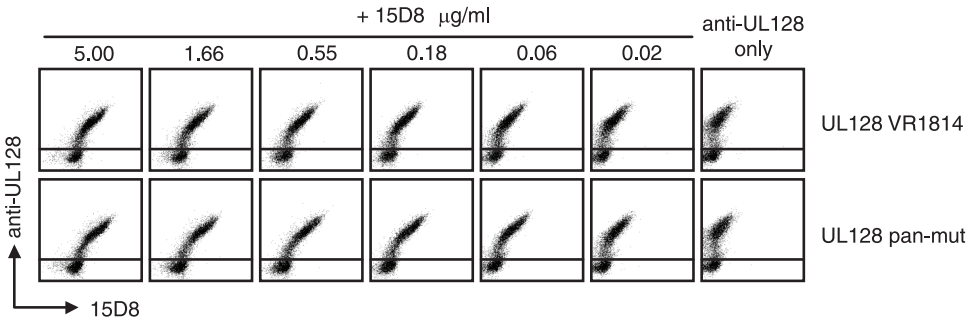

FIG. 3. 15D8 binds to a conserved region of UL128. HEK293T cells expressing wild-type or pan-mutant UL128 were stained with different concentrations of human MAb 15D8 and a noncompeting mouse anti-UL128 MAb. Shown are the dot plots of the two-color staining.

and 6G4 are capable of neutralizing with similar efficiencies different HCMV clinical isolates. This makes monoclonal antibodies specific for conserved epitopes formed by UL128, UL130, or UL131A promising candidates for passive immunotherapy and the corresponding sites on the complexes appealing candidates for a vaccine capable of inducing potent and broadly reactive neutralizing antibodies.

In conclusion, by combining the human memory B-cell immortalization method (59) with the antibody-driven target discovery approach (7), we have been able to exploit the strength of the human antibody response to isolate broadly reactive and potent neutralizing antibodies against HCMV and to identify novel antigenic sites on the gH/gL/UL128-131A complex. The high potency of natural antibodies binding the UL128/130/131A complex and the large number of different epitopes displayed on these proteins suggest a novel subunit vaccine alternative to gB (10). These results illustrate the feasibility of a general approach to study human pathogens and support the value of the "analytic vaccinology" approach in the process of vaccine design (7, 27). Indeed, if the goal of vaccination is to mimic natural immunity by providing native antigen, preparations of vaccine candidates may be examined for their reactivity with neutralizing antibodies, and the response to vaccination may be monitored for elicitation of antibodies with similar binding profiles.

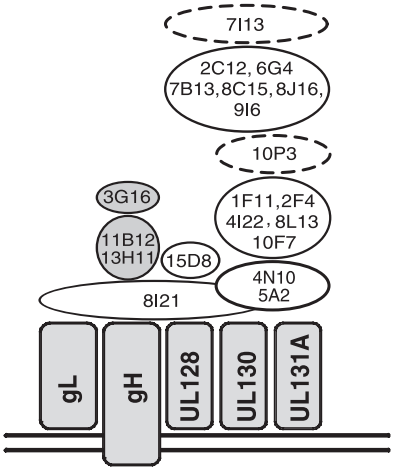

FIG. 4. Antigenic map of gH/gL/UL128-131A complex. Based on minimal antigen requirement for binding and on cross-competition experiments, human monoclonal neutralizing antibodies define nine distinct antigenic sites on gH/gL/UL128-131A. Gray ovals represent sites defined by group 1 antibodies specific for gH, whereas white ovals represent sites recognized by group 2 antibodies. Dashed ovals represent sites defined on the basis of lack of competition by antibodies 7I13 and 10P3 when tested at 100-fold excess.

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