

Neutralization of Human Cytomegalovirus by Human CD13-Specific Antibodies

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The mechanisms of inhibition of cytomegalovirus (CMV) infection by human CD13 (aminopeptidase N)-specific antibodies were studied. These antibodies protect CD13-negative and -positive cells from CMV infection only if incubated with the virus inoculum, suggesting they bind to CMV virions. The association of a CD13-like molecule with virions was further supported by the transfer of CD13 immunoreactivity to the surface of CD13-negative cells upon binding of CMV; the binding of CD13-specific antibodies directly to the surface of CMV virions; and the presence of anti-CD13 immunoreactive bands, including one with mobility similar to that of native cellular CD13 on immunoblots of proteins of purified CMV particles. Importantly, CD13-specific antibodies neutralize CMV in urine of neonates with congenital CMV, indicating that the CD13-like molecule is associated with natural CMV and not acquired *in vitro*. These studies demonstrate that a CD13-like molecule is associated with CMV particles and may be important in CMV pathogenesis.

Human cytomegalovirus (CMV), a herpesvirus, is a significant pathogen in immunocompromised hosts [1–3] that is being seen with increasing frequency in such persons. Despite the availability of antiviral agents, such as ganciclovir and foscarnet, the toxicity of these agents and the development of virus resistance suggests that strategies are needed for prevention and better management of this infection.

CMV has a large double-stranded DNA genome of ~230 kb pairs that provides a potential coding capacity of >200 proteins [4], but little is known about the role of >75% of these genes. Sequences with homology to cellular DNA have been reported within the CMV genome [5–9]. Knowledge of the potential role of these sequences in the pathogenesis of CMV infection could lead to better treatment. A number of host cell-derived molecules are associated with CMV particles [10–14], including molecules associated with the envelope [10–12] and internally within the virion [13, 14]. Immunologic cross-reactivity between a CMV-encoded protein [9] and a

cellular protein and between a CMV virion-associated protein and a host cell membrane protein [10] suggests that CMV-associated molecules with immunologic cross-reactivity with host cell proteins could be important in anti-CMV immunologic reactions and in the pathogenesis of CMV-associated disease [15–18]. This idea is supported by a report in which CD13-specific antibodies were found in CMV-infected bone marrow transplant recipients [17] and in a study in which the antibodies correlated with the development of chronic graft-versus-host disease [18].

We have shown that the expression of human CD13, aminopeptidase (AP) N, on the surface of mouse fibroblasts increases binding and entry of CMV into these cells and that CD13-specific antibodies and inhibitors of AP activity inhibit CMV infection of human fibroblasts [19]. Our initial report did not address whether CD13-specific antibodies inhibited CMV infection by interacting with the host cell or with CMV virions. In the present study, we further characterized the mechanism of inhibition of CMV infection by CD13-specific antibodies and describe some of the complexities involved in these interactions.

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Materials and Methods

Cells and viruses. Human embryonic lung fibroblasts (HL734 and MRC-5) and mouse NIH-3T3 cells were maintained as described [19] in bicarbonate-free MEM with Hanks' salts (GIBCO BRL, Grand Island, NY) supplemented with 25 mM HEPES, 10% fetal bovine serum, glutamine (2 mM), penicillin (50 U/mL), and streptomycin (50 µg/mL) (all from GIBCO). HAPN-3T3 and hAPNMUT-3T3 cells were maintained as above in complete medium with the addition of 800 µg/mL G418 (Geneticin; GIBCO). These cells are NIH-3T3 transfectants that express intact human CD13, hAPN-3T3 [20], or a deletion mutant of human CD13 lacking 39 amino acids (aa) from the active site hAPNMUT-3T3 [21]. The

Table 1. Summary of antibodies used in study of CD13-like molecule associated with CMV virions.

Designation	Specificity	Source	Subclass
Ascites, U81	Human CD13	D. Bourell [24]	IgG2a
Hybridoma supernatants			
7-5	CMV glycoprotein B	W. Britt [25]	IgG1
WM47	Human CD13	Dako (Carpinteria, CA) [26, 27]	IgG1
8A3.1	Human CD13	T. Piela-Smith [28]	IgG1
7B.1	Human CD13	T. Piela-Smith	IgG1
RBS	Human CD13	K. Holmes [29]	ND
Purified immunoglobulins			
3D8	Human CD13	Caltag Laboratories (South San Francisco) [26]	IgG2a
L138 (anti-Leu M7)	Human CD13	Becton Dickinson (San Jose, CA) [26]	IgG1
MY7	Human CD13	Coulter Immunology (Hialeah, FL) [27, 30]	IgG1
SJ1D1	Human CD13	ImmunoTech (Westbrook, ME) [26]	IgG1
22A5	Human CD13	Leinco Technologies (Ballwin, MO) [30]	ND
WM15	Human CD13	Pharmingen (San Diego) [26, 27]	IgG1
1B10	Human fibroblast surface protein	Sigma (St. Louis) [31]	IgM
MφP-9	Human CD14	Becton Dickinson [32]	IgG2b
FMC 8	Human CD9	SeraLab (Crawley Down, UK)	IgG2a
IVIG	Pooled human immunoglobulins	J. Hooper [34]	
MOPC21	Isotype control	Sigma	IgG1
UPC10	Isotype control	Sigma	IgG2a

NOTE. ND, not done.

human glioblastoma-derived cell line (U373MG [ATCC HTB 17]; American Type Culture Collection, Rockville, MD) was maintained in bicarbonate-free MEM as described above. The Hyb7.2 cells (obtained from L. K. Vogel, Panum Institute, University of Copenhagen) are MDCK transfectants that secrete a mutant form of human CD13 corresponding to aa 65–967. This form of CD13 lacks the transmembrane domain and exists in soluble form [22]. The Hyb7.2 cells were maintained as described for the hAPN-3T3 cells.

CMV strains AD169 and RC256, a recombinant Towne strain with the *Escherichia coli* β -galactosidase (β -gal) gene under the control of the CMV major early gene promoter [23], were propagated in human embryonic lung fibroblasts grown in complete medium, and titers were determined as described [19]. Urine specimens from neonates with congenital CMV infection known to be viruric (provided by J. Rabalais, Kosair Children's Hospital, Louisville) were stored by 1:1 dilution in 50% sucrose, 12 mM K_2HPO_4 , 7.5 mM KH_2PO_4 at $-70^\circ C$ until use. The virus titer in urine samples was determined by expression of the major immediate early (mIE) protein (UL123) as described for the CMV laboratory strains [19].

Immunologic reagents. We used a panel of 11 different human CD13-specific mouse monoclonal antibodies (MAbs) and various control antibodies in the form of ascites, hybridoma supernatants, or purified mouse immunoglobulin. The source and isotype, if known, of each antibody used is listed in table 1. The characterization of the interactions of the CD13-specific MAbs with human CD13 has been described [19, 24, 26–30]. It has been suggested that MAbs directed toward CD13 recognize as many as 7 to 9 different epitopes defined by their ability to block APN activity, bind to mouse transfectants expressing a mutant form of CD13, or block binding of other CD13-specific MAbs [24, 35]. The CD13-

specific MAbs used in this study recognize at least three different epitopes according to this criterion.

Before any antibodies were used in the CMV neutralization assay (see below), they were dialyzed extensively against water and concentrated using a microdialyzer apparatus and concentrating solution (Pierce, Rockford, IL). The IgG concentration of the stock solutions was determined by ELISA (see below). Immunocytochemical stain for detection of the CMV mIE protein used a purified mouse IgG, E13 (ARGENE, Varilhes, France) or 9221 (NEN DuPont, Boston).

An immunoglobulin fraction from pooled serum from CMV-seropositive persons (intravenous immune globulin; IVIG) [34] and a CMV glycoprotein B (gB)-specific mouse MAb (7-5) [25] were used throughout this study as positive controls. IVIG has a high neutralizing titer toward CMV [36] and recognizes a number of proteins in lysates from CMV-infected cells by both immunoprecipitation [36] and immunoblot analysis [37] (including the products of CMV UL99 pp28 [37] and UL83 pp65 genes [data not shown]). The CMV neutralizing activity of serum from seropositive persons is primarily directed against gB [38, 39]. The gB-specific MAb 7-5 has high neutralizing activity [25]. IVIG and MAb 7-5 do not react with human CD13 in immunoblot analysis or bind to mouse cell transfectants expressing human CD13 (data not shown).

Determination of IgG concentration. IgG concentrations of the MAbs were determined quantitatively by a sandwich ELISA [40]. Briefly, 96-well EIA plates (Costar, Cambridge, MA) were coated overnight at room temperature with affinity purified goat anti-mouse or anti-human IgG (2 $\mu g/mL$; both from Sigma, St. Louis) in carbonate buffer. Plates were then incubated overnight at room temperature with a dilution series of unknown mouse MAbs or IVIG plus a standard mouse or human IgG (Sigma). The plates

were incubated with an alkaline phosphatase-conjugated goat anti-mouse or anti-human IgG (both from Sigma) for 1 h at room temperature. Finally, the substrate *p*-nitrophenyl phosphate (1 mg/mL; Sigma) in ethanolamine, pH 9.7, was added and the free *p*-nitrophenol was determined at 410 nm by microplate reader (MR600; Dynatech Laboratories, Alexandria, VA). Between each incubation step, plates were washed four times with PBS containing 0.05% Tween 20. Absorbance readings were converted to IgG concentrations using Excel software (Microsoft, Seattle).

Inhibition of CMV infection by CD13-specific MAbs. The neutralization of CMV infection of MRC-5 or U373MG cells by CD13-specific MAbs was assayed as described [19]. In brief, dilutions of the test antibody and CMV were incubated in binding buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg/mL bovine serum albumin [BSA], 1 mg/mL glucose, and 10 µg/mL phenol red) for 30–60 min at 4°C before being added to the cells. Monolayer cultures were incubated with virus alone or with virus and antibody for 2 h at 4° or 37°C. After incubation with virus, the cells were washed in complete medium, incubated at 37°C for 16–20 h, and assayed for CMV infection as described [19] by detection of expression of either the CMV mIE protein or β-gal if cells were infected with CMV RC256.

To test the ability of CD13-specific MAbs to neutralize unpassaged wild CMV, urine specimens from neonates with congenital CMV infection were used as a source of CMV. The specimens were stored in 25% buffered sucrose at –70°C until use. Samples were titered and 100–200 infectious units were treated at 37°C for 1 h with 100 µg/mL CD13-specific MAb L138 or mouse IgG1, the isotype control, and cultured in 96-well plates on MRC-5 monolayers for 18 h. The cells were assayed for infection by measuring the expression of mIE protein as described above.

Transfer of a CD13 immunoreactivity to CD13-negative cells. After NIH-3T3 cells were scraped from culture flasks into PBS and washed twice in PBS, 5 × 10⁵ cells were incubated with 1.5 mL of a CMV inoculum (5 × 10⁶ pfu/mL) for 30 min at room temperature. Infected or uninfected cells were then incubated with either a MAb directed toward CD13 (L138), CMV gB (7-5), the human fibroblast surface antigen (1B10), CD14 (MφP-9), CD9 (FMC 8), or mouse IgG for 25 min at 4°C, followed by incubation with a phycoerythrin-conjugated F(ab')₂ fragment of a rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) for 20 min at 4°C and finally resuspended in PBS and analyzed by flow cytometry as described below. Cells were washed twice with ice-cold PBS between each incubation step.

Flow cytometry of cell surface CD13. The fluorescence signal from 5 × 10⁴ MRC-5, U373MG cells, hAPNMUT-3T3 cells, or 10⁴ uninfected or infected NIH-3T3 cells was determined by flow cytometer (FACSsort; Becton Dickinson, San Jose, CA) with an argon laser producing 15 mW of light at 488 nm as described [19]. For comparison of CD13 levels on the surface of MRC-5 fibroblasts and U373MG and hAPNMUT-3T3 cells, frequency distribution profiles of log₁₀ fluorescence of samples labeled with anti-CD13 (L138) or the isotype-matched control (MOPC21) were plotted, and a modal value of 1.0 was assigned to cells stained with the isotype-matched control. Dead cells labeled with propidium iodide (0.5 µg/mL) were excluded from analysis.

For the NIH-3T3 cells, the frequency distribution profiles of log₁₀ fluorescence for uninfected and infected cells were compared for each antibody tested. A positive signal, defined by Kolmo-

gorov-Smirnov statistics [41], was an increase in the mean red fluorescence of NIH-3T3 cells greater than 10 channels when CMV-infected cells were compared with uninfected cells.

Immunogold labeling and electron microscopy of CMV virions. Aliquots (150 µL) of culture supernatants from AD169-infected HL734 fibroblasts were incubated 1 h at 23°C with 0.3 µg of CD13-specific MAb (L138), 12 µg of total mouse IgG, 5 µg of CD10-specific MAb (SS2/36), or 6.6 µg of CMV gB-specific MAb (7-5). A colloidal gold (10 nm)-conjugated goat anti-mouse IgG (Sigma) was added, and incubation was continued 1 h at 23°C. Virus particles were spun directly using a Microvette (Sarstedt, Landskrona, Sweden) onto formavar- and carbon-coated 400-mesh copper grids (Gilder Grids, Grantham, UK). Samples were examined by electron microscope (Philips CM 100) using 2% phosphotungstic acid as negative stain. The concentration of the CD13- and CMV gB-specific MAbs was determined empirically based on the concentration that minimized formation of aggregates of virions, dense bodies, and gold particles. To quantitate the number of gold particles associated with CMV particles after labeling with the various MAbs, the specimen relocation system of the microscope was used to randomly select areas for gold particle counting. Specimens were studied at ×25,000, and all virus particles were analyzed, including dense bodies in the preselected microscopic view. We counted 100 particles for each condition and determined the percentage of virus particles labeled with gold. Virus particles that were gold particle-positive were tallied according to the number of particles associated with each individual virus particle.

Analysis of CMV virion proteins by SDS-PAGE and immunoblot. CMV virions and dense bodies were purified from media of infected human fibroblasts using negative viscosity and positive-density gradients as described by Talbot and Almeida [42]. Briefly, virus particles were collected from media of infected MRC-5 fibroblasts by centrifugation of cleared supernatants at 120,000 g (SW-28 rotor; Beckman, Brea, CA) for 45 min at 4°C through an 8-mL 20% sucrose cushion. The pellet was resuspended in PBS, sonicated, layered on a 20%–70% sucrose gradient, and centrifuged as above. Banded particles were collected, concentrated by centrifugation, layered onto a potassium tartrate-glycerol gradient formed as described by Talbot and Almeida [42], and centrifuged at 260,000 g (SW-41 rotor; Beckman) for 18 h at 4°C. The enveloped virion and dense body bands were collected, concentrated by centrifugation, resuspended in PBS, and stored at –70°C.

NIH-3T3 and hAPN-3T3 cell and MRC-5 fibroblast membranes were collected by scraping cell monolayers into TRIS-buffered saline (TBS; 20 mM TRIS-HCl, pH 7.2, 150 mM NaCl), pelleted by centrifugation (IEC Centra-7R; Damon/International Equipment, Needham Heights, MA) at 800 g for 10 min, and homogenized with 15 strokes in a Dounce homogenizer in TBS containing 4 µg/mL aprotinin, leupeptin, and soybean trypsin inhibitor (all from Sigma). The homogenate was centrifuged as above (800 g, 10 min), and the pellet was rehomogenized and centrifuged. Membranes were purified from the combined supernatants by centrifugation at 120,000 g in a Beckman SW-28 rotor for 45 min at 4°C. The membrane pellets were resuspended in TBS and stored at –70°C. Protein concentrations were estimated by the bicinchoninic acid method [43]: BSA was the standard and reagents were used according to the manufacturer's (Pierce) instructions.

Cellular membrane protein (10 µg) and protein from purified enveloped CMV virion and dense body preparations (30 µg) were

Table 2. CD13 antibody characterization.

Antibody clone	Inhibition of CMV infection*	Inhibition of APN activity†	Binding to CD13 deletion mutant‡
L138	+++	-	+
8A3.1	+++	-	+
7B.1	++	-	+
MY7	++	+	+
SJ1D1	+	-	+
RBS	+	++	-
3D8	-	+	+
U81	-	++	-
22A5	-	-	+
WM15	-	++	-
WM47	-	-	+

* -, <25%; +, 25%–50%; ++, 50%–70%; +++, >70%.

† Inhibition of aminopeptidase N (APN) activity associated with surface of NIH-3T3 transfectants expressing human CD13: -, <40%; +, 40%–60%; ++, >60%.

‡ Ability of antibody to bind to NIH-3T3 transfectants expressing mutated form of human CD13 lacking 39 amino acids from active site.

separated by SDS-PAGE on 6.0% gels according to the method of Laemmli [44] and processed for immunoblot. For immunoblot analysis, proteins were transferred electrophoretically from the gel to a nitrocellulose membrane (BioRad, Richmond, CA) as described [37]. The immunoreactive bands were detected by incubating the nitrocellulose membrane with a CD13-specific MAb (3D8; 5 μ g/mL), the isotype control (UPC10; 5 μ g/mL), or the CD13-specific MAb preabsorbed with 10 μ g/mL of a soluble form of human CD13, followed by a biotinylated goat anti-mouse IgG (Sigma) and a horseradish peroxidase-conjugated streptavidin (Boehringer Mannheim, Indianapolis). Finally, bands were visualized by incubation of the nitrocellulose membranes with the chemiluminescence substrate luminol (Boehringer Mannheim) and exposed to Kodak X-OMAT AR-5 film according to the manufacturer's instructions. Molecular standards used to calibrate immunoblots were from a mix of prestained proteins (BioRad) that contained myosin (200 kDa), phosphorylase b (110 kDa), BSA (84 kDa), ovalbumin (47 kDa), carbonic anhydrase (33 kDa), and soybean trypsin inhibitor (24 kDa).

A soluble form of human CD13, 65–967 aa, which lacks the N-terminal end that contains the transmembrane domain [22], was purified from media of hyb7.2 cells using standard biochemical techniques (unpublished data). This protein has AP activity and is reactive with the CD13-specific MAbs used in this study.

Results

Characterization of CMV neutralization by cell surface antigen-specific MAbs. To characterize the inhibitory effect of CD13-specific MAbs on CMV infection, MRC-5 fibroblasts were incubated concurrently with MAb and virus inoculum (see Materials and Methods). We initially found that all CD13-specific MAbs inhibited CMV infection (data not shown); however, further characterization showed that the salt content of

some antibody preparations was sufficient to inhibit CMV (data not shown). To avoid this, the MAbs were dialyzed into water and concentrated and assayed in parallel with a dialyzed salt control. We tested the ability of 11 different CD13-specific MAbs to protect MRC-5 fibroblasts from CMV infection (table 2). Four MAbs strongly inhibited CMV infection, 2 had weak inhibitory activity, and 5 had none.

Figure 1 shows the three levels of inhibitory activity of the CD13-specific MAbs and of antibodies directed toward two other cell surface antigens (CD9 and the epidermal growth factor receptor), IVIG used as a positive control, and the isotype controls. If the cells were infected in the presence of the CD13-specific MAbs, CMV infection was inhibited in varying degrees: 8A3.1 inhibited CMV to 23.6% \pm 4.5% (mean \pm SE) of the level of the control, SJ1D1 inhibited CMV to 57.5% \pm 6%, and 3D8 had no inhibitory activity (100.8% \pm 9.7%; figure 1). The positive control, IVIG, inhibited infection to 36.4% \pm 2%; the isotype controls, IgG1 and IgG2a, and the antibodies directed toward CD9 and the epidermal growth factor receptor had no effect on CMV infection (figure 1). These results indicate that the antiviral activity of CD13-specific MAbs is not merely due to interactions with an Fc receptor, to nonspecific binding, or to binding to any antigen on the cell surface.

Antibodies capable of inhibiting CMV infection can be placed in three groups according to their ability to bind to NIH-3T3 transfectants that express a mutated form of human CD13 lacking 39 aa from the active site (hAPNMUT-3T3) [21] and

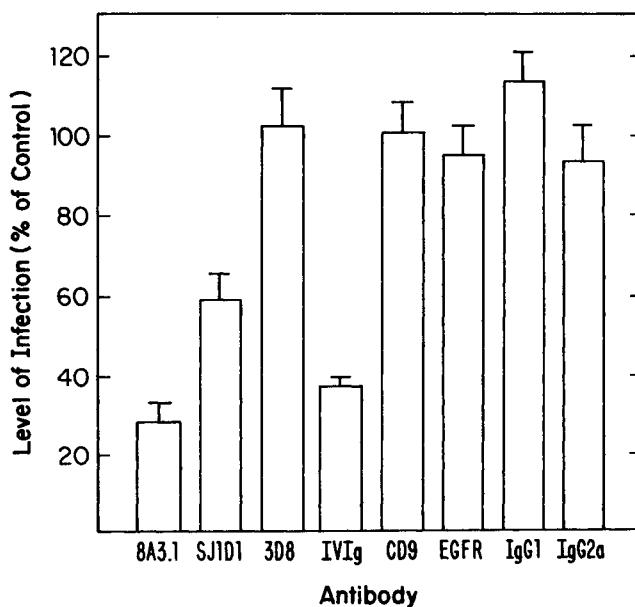


Figure 1. Inhibition of CMV infection of human embryonic lung fibroblasts by antibodies to human CD13. Antibodies were tested for ability to protect MRC-5 fibroblasts from CMV RC256 infection by coinubation with CMV inoculum during infection. 8A3.1, SJ1D1, and 3D8 are CD13-specific monoclonal antibodies. All antibodies were used at 100 μ g/mL final concentration. Infection level is expressed as % of dialyzed buffer control (mean \pm SE).

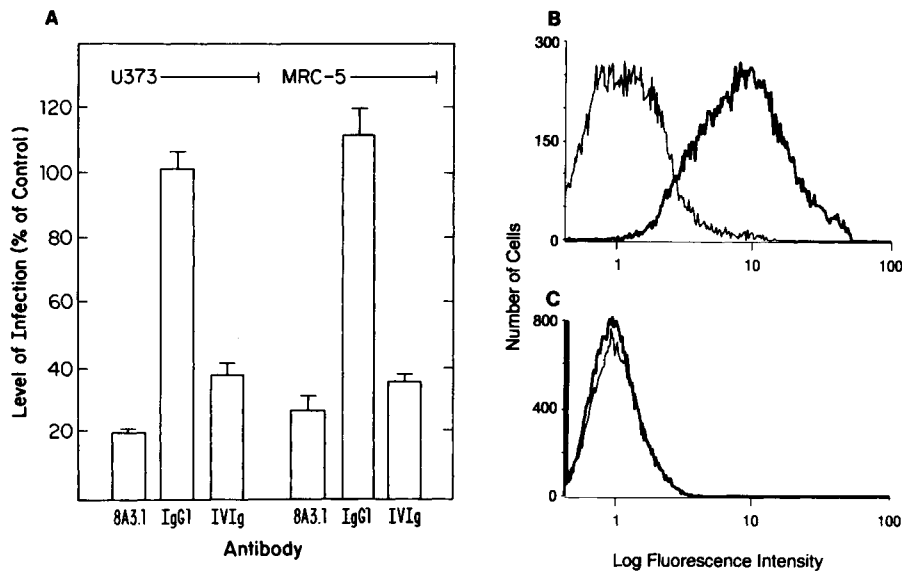


Figure 2. Inhibition of CMV infection of CD13-negative cells with antibodies to human CD13. **A**, Level of CMV infection of monolayer cultures of U373MG cells or MRC-5 fibroblasts, if infected, in presence of CD13-specific antibody 8A3.1; isotype-matched control, IgG1; or immunoglobulin fraction from pooled CMV-seropositive persons (IVIg), all at 100 $\mu\text{g}/\text{mL}$. Infection level is expressed as % of dialyzed buffer control (mean \pm SE). **B**, **C**, Flow cytometry of surface expression of CD13 in MRC-5 fibroblasts and U373MG cells, respectively. Fluorescence levels are shown after binding of CD13-specific monoclonal antibody (L138, thick line) or isotype-matched control antibody (IgG1, thin line).

inhibit APN activity of NIH-3T3 transfectants that express native human CD13 (hAPN-3T3) [20] (table 2). The three antibody groups are as follows: those that bind to hAPNMUT-3T3 cells and inhibit APN activity (e.g., MY7), those that bind to hAPNMUT-3T3 cells but do not inhibit APN activity (e.g., L138, 8A3.1, 7B.1, and SJ1D1), and those that do not bind to the deletion mutant but do inhibit APN activity (e.g., mouse MAb RBS).

When the MRC-5 fibroblasts were incubated with antibody and excess antibody was washed away before infection with untreated inoculum, none of the antibodies tested inhibited CMV infection (data not shown). These results suggest that CD13-specific MAbs inhibit CMV infection by an interaction with the virus rather than with the host cell.

CD13-specific MAbs protect CD13-negative cells from CMV infection. To test whether CD13-specific MAbs interact with CMV per se, we studied the effect of these MAbs on infection of CD13-negative cells. U373MG and MRC-5 cells were infected with CMV RC256 (figure 2A) in the presence of CD13-specific MAb 8A3.1, the isotype-matched negative control (IgG1), or IVIG (positive control). Infection of MRC-5 and U373MG cells by CMV was inhibited both by the CD13-specific MAb and by IVIG. The isotype-matched control for the CD13-specific MAb showed no protective effect. Flow cytometry of these cells (figure 2B, C), using either a CD13-specific MAb or the isotype-matched control, confirmed that the U373MG cells (figure 2B) had no detectable binding of the CD13-specific MAb but that the MRC-5 cells (figure 2C) had significant binding of the CD13-specific MAb. We have tested whether CD13-specific MAbs can protect CD13-negative mouse cells [19] from CMV infection. Like the U373MG cells, the NIH-3T3 cells also were protected by a CD13-specific MAb and IVIG but not by the isotype-matched control (data not shown). Since the CD13-specific MAb does not bind to

the U373MG or NIH-3T3 cells, the neutralization of CMV by this MAb suggests that it reacts directly with an epitope on the CMV virion.

Transfer of a virus-associated CD13-like molecule to CD13-negative cells. To confirm that a CD13-like molecule is associated with CMV virions, we indirectly measured the presence of cell-surface molecules associated with the CMV envelope by comparing the surface immunoreactivity of human CD13-negative murine cells before and after CMV binding. NIH-3T3 cells were analyzed by flow cytometry for the presence of human cell surface antigens, including CD13, and CMV gB before or after incubation with CMV. Flow cytometry of the uninfected NIH-3T3 cells showed no binding of the human CD13-specific MAbs L138 (figure 3C) or WM47 (data not shown) or of the anti-CMV gB-specific MAb 7-5 (figure 3E). The NIH-3T3 cells showed a marked increase in the binding of anti-CMV gB MAb, detected as an increase in mean fluorescence, after incubation with CMV (figure 3E, F [shaded areas]), indicating that CMV gB was associated with the NIH-3T3 cells, presumably by binding CMV virions. The binding of CMV to the NIH-3T3 cells resulted in a significant increase in binding of both CD13-specific MAbs L138 and WM47 (figure 3C, D [shaded areas], and data not shown). The negative control antibodies (1 directed toward CD14 [M ϕ P-9] and another to mouse IgG) did not bind to the NIH-3T3 cells before or after CMV binding (figure 3A, B [open tracing], and data not shown). The binding to the NIH-3T3 cells by 2 other antibodies specific for the cell surface proteins CD9 (FMC 8) and a human fibroblast surface antigen (1B10), both present on MRC-5 fibroblasts, was not affected by incubation of the cells with CMV (data not shown). The binding of CMV to a cell results in transfer of human CD13 immunoreactivity, suggesting that a CD13-like molecule is associated with CMV particles.

Immunogold labeling of CMV. To test directly if CD13-specific MAbs interact with CMV, MAbs were assayed for their

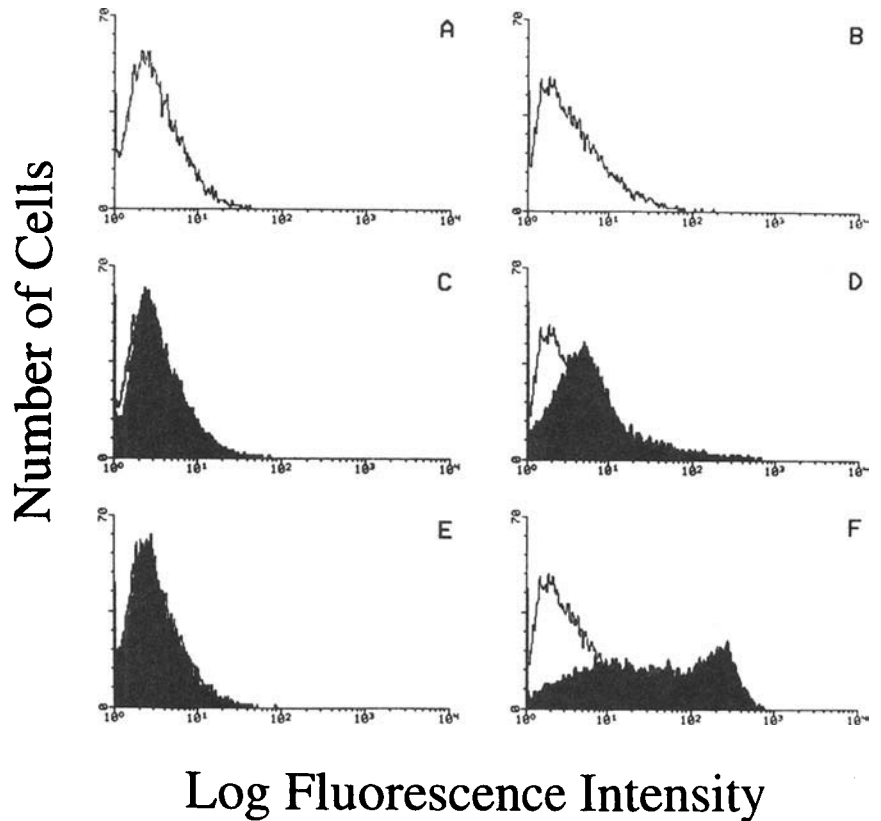


Figure 3. Flow cytometry of CD13 and CMV glycoprotein B (gB) on NIH-3T3 cells after infection with CMV. NIH-3T3 cells were stained with CD13-specific monoclonal antibody (MAb) L138 (C, D, shaded area), CMV gB-specific MAb 7-5 (E, F, shaded area), or mouse IgG (A–F, open area) before (A, C, E) or after (B, D, F) CMV infection.

ability to bind to virions by immunogold labeling of virions and analyzed by electron microscopy (see Materials and Methods). Significantly more gold particles bound to virion envelopes (figure 4) and dense bodies (data not shown) when the primary MAb was either CMV gB-specific (figure 4A) or CD13-specific (figure 4D) than when the primary antibody was CD10-specific or mouse IgG (figure 4C, B, respectively). The number of gold particles associated with CMV particles was quantitated as described in Materials and Methods. When we compared the number of gold particles associated with 100 CMV particles labeled by each of the primary antibodies (table 3), we found that virus particles labeled with the negative mouse IgG control usually had 0–2 gold particles on their surfaces. Therefore, we considered that 0–2 gold particles indicated a nonspecific interaction of the primary antibody with virus particles.

Gold particles were associated with 67% of virus particles labeled with antibody specific to CMV gB compared with 40% of virus particles incubated with a CD13-specific MAb (table 3). Only 1% of the particles labeled with mouse IgG or the CD10-specific MAb had >2 gold particles associated with them. As shown in table 3, virions incubated with CD13-specific MAbs had significantly fewer associated gold particles than virions incubated with the gB-specific MAb, (3.07 ± 2.42 vs. 4.47 ± 2.88 [mean \pm SD]; $P = .0013$). The CD13-specific MAb-labeled CMV particles had significantly more gold particles bound than did virus particles labeled with a CD10-specific

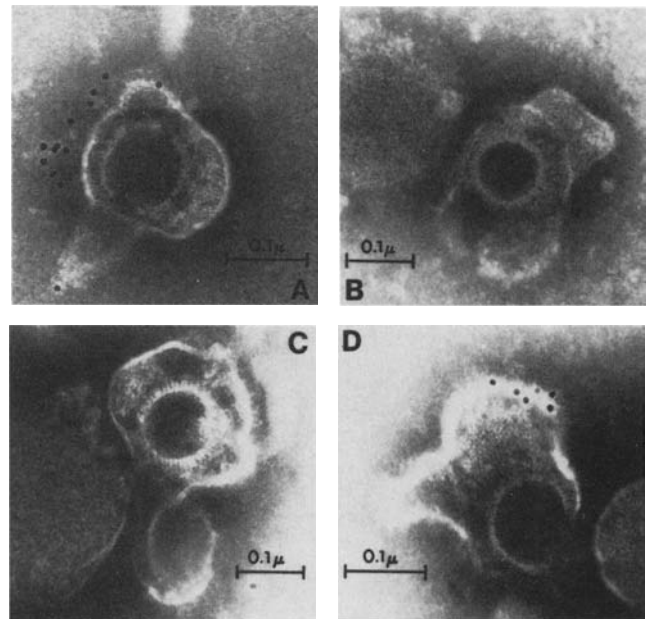


Figure 4. Immunogold labeling of CMV virions with CD13-specific antibodies. Aliquots of AD169 inoculum were incubated with CMV glycoprotein B-specific antibody (A), mouse IgG (B), CD10-specific antibody (C), or CD13-specific antibody (D) followed by gold-conjugated secondary antibody. Samples were analyzed by transmission electron microscopy. Magnification: $\times 230,000$ (A, D), $\times 190,000$ (B, C).

Table 3. Immunogold labeling of CMV particles with CD13-specific monoclonal antibodies.

Antibody	No. of antibody linked gold particles bound to CMV particles*				% CMV particles labeled with gold particles [†]	No. of gold particles associated with CMV particles [‡]
	0-2	3-5	6-8	9-15		
mIgG	99	1	0	0	1	1.42 ± 0.55
CD10	99	1	0	0	1	1.35 ± 0.52
CD13	60	34	3	3	40	3.07 ± 2.42
Glycoprotein B	33	38	22	7	67	4.47 ± 2.88

* CMV particles grouped by no. of associated gold particles.

[†] % CMV particles labeled with >2 gold particles.

[‡] No. of gold particles associated with CMV particles (mean ± SD).

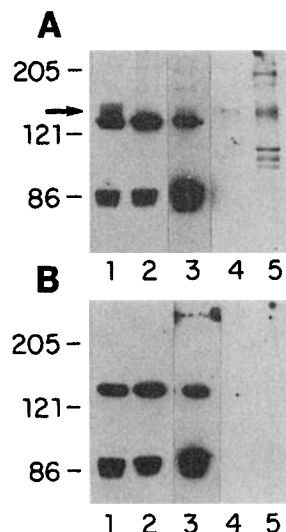
MAB (1.35 ± 0.52; $P = .0001$) or a mouse IgG (1.42 ± 0.55; $P = .0001$). The CD10-specific MAB used for these experiments is the same isotype as the CD13-specific MAB, indicating that the interaction of the CD13-specific MAB is not merely via the Fc receptor. These experiments show that CD13-specific MABs bind directly to CMV virions and dense bodies.

Immunoblot analysis of CD13-like molecules associated with CMV virions. In an attempt to determine which CMV molecules are immunoreactive with CD13-specific MABs, we analyzed immunoblots of purified virion proteins. CMV virions and dense bodies were purified using negative viscosity: Positive density gradients [42] and virus particle proteins were fractionated by SDS-PAGE and assayed for immunoreactivity to a CD13-specific MAB (3D8; figure 5A) and the isotype control (UPC10; figure 5B). The CD13-specific MAB was immunoreactive with as many as seven bands in the fractionated proteins of CMV dense bodies (figure 5A, lane 5). The major band (apparent molecular mass, 150 kDa) was also visible in fractionated proteins from CMV-enveloped virions along with a cluster of three very faint 95- to 115-kDa bands (figure 5A,

lane 4). The 150-kDa immunoreactive band in CMV virions and dense bodies has a similar apparent molecular mass by SDS-PAGE to that of native human CD13 expressed in hAPN3T3 cells (figure 5A, lane 1 arrow) and faintly evident in membranes from MRC-5 fibroblasts (figure 5A, lane 3). The other major immunoreactive bands at 137- and 86-kDa in the cell membrane fractions recognized by the CD13-specific MAB (figure 5A, lanes 1-3) were also recognized by the isotype control antibody (figure 5B, lanes 1-3), indicating a nonspecific interaction. If the CD13-specific MAB is preabsorbed with a soluble form of CD13 purified from hyb7.2 cell media [22] as described in Materials and Methods, only the 137- and 86-kDa bands are visible on the immunoblot (data not shown). These immunoblot data indicate that CD13-specific MABs recognize a number of proteins, including a 150-kDa protein, that are associated with purified virions and dense bodies.

Inhibition of natural unpassaged CMV with CD13-specific MABs. Because laboratory CMV strains are generally propagated in CD13-positive human fibroblasts, it is possible that the introduction of CD13 to the virion is a tissue culture artifact. To test whether a CD13-like molecule is associated with CMV in nature, we used previously frozen and virus-titered urine samples of neonates with congenital CMV infection. A total of 100-200 infectious units of such unpassaged virus were incubated with a mixture of 2 CD13-specific MABs (8A3.1 and 7B.1) and cultured with MRC-5 cells. Fibroblast infection by natural CMV was inhibited 89% by CD13-specific MABs (table 4) and not at all by the isotype control. The clinical sample and laboratory strain AD169 (diluted and tested in urine from a seronegative female volunteer) were similarly inhibited (89% and 98%, respectively). Thus, CD13-specific MABs inhibit natural unpassaged CMV, indicating that a CD13-like molecule is associated with natural and clinically relevant CMV strains and not merely a laboratory artifact derived by culturing virus in CD13-positive cells.

Figure 5. CD13 immunoreactive material associated with purified CMV particles. Membrane fractions from hAPN3T3 cells (lane 1) that express human CD13, NIH-3T3 cells (lane 2), MRC-5 fibroblasts (lane 3), CMV virions (lane 4), and dense bodies (lane 5) were analyzed by immunoblotting using CD13-specific antibody 3D8 (A) or IgG2a isotype control UPC10 (B). Arrow indicates 150-kDa immunoreactive band at mobility expected for native CD13.



Discussion

In the current study, we extended our initial observations that CD13-specific MABs inhibit CMV infection by showing

Table 4. Inhibition of natural, unpassaged CMV with CD13 antibody.

Specimen	Antibody	Level of infection %
829N	anti-CD13	12 (7, 16)
	IVIG	69 (74, 63)
	IgG1	121 (130, 112)
AD169	anti-CD13	3 (2, 3)
	IVIG	13 (14, 12)
	IgG1	100 (98, 102)

NOTE. CD13-specific monoclonal antibody, isotype negative control, or IVIG (intravenous immune globulin) as positive control were tested for ability to neutralize CMV in urine specimen from neonate. Level of infection is shown as % of level in control infected in presence of equivalent volume of dialysed buffered saline. Values listed are mean of 2 separate determinations; values for individual determinations are in parentheses.

that the inhibition is caused by an interaction between the MABs and virion-associated epitopes. The interaction between MABs and virus appears to be via an antigenic molecule that is physically attached to CMV as shown by immunogold labeling with CD13-specific MAB and by the transfer of CD13 immunoactivity upon binding of CMV to CD13-negative cells. A number of antigenic molecules are associated with purified virions and dense bodies, including one with similar molecular mass (by immunoblot analysis) to native human CD13. Natural unpassaged CMV is similarly neutralized by CD13-specific MABs, showing that this is not merely a laboratory artifact acquired by passage in human fibroblasts. As shown previously [19], CD13-specific MABs do not inhibit infection by a closely related member of the herpesvirus family, herpes simplex virus type 2. Thus, neutralization of CMV by anti-CD13 is specific and suggests that a CD13-like molecule associated with CMV plays an important role in CMV infection [45].

Although the role of CD13 in CMV infection is incompletely understood, the results of this study and our previous findings [19] suggest that interactions between the host cell and the virus involve CD13 in a way that enhances infection. The data from the present study provide some information about these interactions. Our prior study showed that expression of human CD13 on the surface of a mouse cell increases the susceptibility of the cells to human CMV infection. This suggested that cellular CD13 (cCD13) interacts with a virion protein. The present study shows that CD13-specific MABs can neutralize CMV infection by interacting with CD13 or a CD13-like molecule associated with virions, but that the MABs do not interfere with infection when bound to the cell surface. The effect of these MABs on CMV can be explained by at least three hypotheses: first, that antibody binds to the cell and virus via the same epitope that exists in different proteins and that the CD13-like molecule associated with CMV is not CD13; second, that antibody binds to CD13 on the cell and on the virus, but the interactions of these proteins necessary for enhancing infection involve different ligands for the cCD13 compared with viral

CD13; and third, that the antibody binds to CD13 on the cell and virus, and the proteins interact with the same or a homologous protein that exists on the virus and the cell.

Our data disprove the third hypothesis, since antibody bound to the cell surface does not inhibit CMV infection. The data do not exclude the other two hypotheses. Not all CD13-specific MABs neutralize infection; however, at least 2 MABs that lack neutralizing activity, 3D8 and WM47, bind to CMV virions as shown by immunoblot and FACS analyses after the transfer of immunoreactivity by binding of CMV virions to CD13-negative cells (figure 5; data not shown). In fact, these data indicate that at least 8 of 11 different MABs used in this study interact with CMV virions. The ability of these MABs to inhibit CMV infection and APN activity or to bind to the mutated form of CD13 (table 2) suggests that these 8 antibodies recognize as many as 5 different CD13 epitopes and supports the second hypothesis that the immunoreactive protein associated with CMV particles is actually cCD13.

We have compared the cDNA-deduced protein sequence for human CD13 [20] to the deduced protein sequences of the known and suggested open-reading frames encoded by the CMV AD169 genome [4]. There were no long stretches of significant sequence homology to human CD13 within the CMV genome, indicating there is no CMV homologue to human CD13. However, this analysis identified a number of short peptide sequences (5–11 aa long) from the deduced sequence of putative CMV glycoproteins. These short homology sequences could represent linear epitopes or regions of discontinuous epitopes to which these antibodies bind [45, 46]. For example, Fujinami et al. [9] showed that a peptide from a protein encoded by the CMV IE-2 region, containing 5 aa, is identical to an HLA-DR β chain peptide and induces antibodies that cross-react with HLA-DR [9]. Thus, it is possible that a peptide from some CMV surface protein is similar in sequence to a natural CD13 epitope.

Our current working hypothesis is that cCD13 and a viral CD13-like molecule interact with a viral or cellular protein, respectively, and that both interactions enhance viral infection. How then does this hypothesis explain that CD13-negative cells, such as U373MG (see figure 2), can be infected with CMV [47, 48]? Although we do not know the answer, we believe there are at least two possible explanations: First, cCD13 is important for CMV infection but not the only mechanism of CMV uptake and it is not essential; and second, in CD13-negative cells, another mechanism of uptake predominates, but in CD13-positive cells, the CD13-mediated uptake is a supplementary method that enhances CMV uptake. Other enveloped viruses have multiple entry pathways [49, 50]. For example, human immunodeficiency virus can infect CD4-negative cells via pathways involving sphingolipid galactosyl ceramide or the Fc receptor (reviewed in [49]). Further study is needed to determine if CD13 or some homologue is necessary for CMV infection. A number of cell surface molecules play a role in the initial events of CMV infection of fibroblasts

(summarized in [51]). How CD13 interacts with these proteins remains to be determined.

This study shows that CMV from natural infection has a CD13-like molecule associated with infectious particles. This suggests that a better understanding of the physical characteristics of the CD13-like molecule(s) associated with CMV particles, including the sequences recognized by CD13-specific MAbs that neutralize CMV, might increase our understanding of CMV infection, disease, and pathogenesis. In this regard, it is important to consider that a CD13-like molecule associated with CMV might play an important role in the immunologic response of the host to CMV. Immune responses initiated towards viral antigens sharing homology with host cell proteins can lead to autoimmune responses [52, 53]. Our recent data show that some bone marrow transplant patients with CMV disease or infection have cytotoxic antibodies with specificity toward CD13 [17]. The production of such autoantibodies could contribute to tissue damage during graft-versus-host reactions in the bone marrow transplant patient [18] or to graft rejection in the solid organ transplant recipient. Whether the CD13-like molecule associated with CMV virions is cellular CD13 or an immunologically related molecule, this association should be considered in the elucidation of interactions of CMV pathogenesis.

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