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Anti-Idiotype Antibodies That Mimic gp86 of Human Cytomegalovirus Inhibit Viral Fusion but Not Attachment

SUSAN KEAY[1,2]* AND BRENDA BALDWIN[2]

Research Service, The Department of Veterans Affairs Medical Center,[1] and Division of Infectious Diseases, Department of Medicine, University of Maryland School of Medicine,[2] Baltimore, Maryland 21201

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Human cytomegalovirus (CMV) infects cells by sequential processes involving attachment, fusion with the cell membrane, and penetration of the capsid. We used two monoclonal anti-idiotype antibodies that mimic one of the CMV envelope glycoproteins, gp86, to study its role in the early phases of CMV infection. Neither of two such antibodies inhibited virus binding to human embryonic lung (HEL) fibroblasts; however, both antibodies inhibited the fusion of CMV with HEL cells, as measured by an assay in which viral envelope is labeled with a fluorescent amphiphile (octadecyl rhodamine B chloride, or R_{18}), resulting in increased fluorescence during fusion of virus with the cell membrane. Because these anti-idiotype antibodies were shown previously to bind to specific receptors on HEL cell membranes, these findings suggest that both gp86 and its cell membrane receptor may function in the fusion of human CMV with HEL cells.

Human cytomegalovirus (CMV) is a member of the herpesvirus group that causes disease in nonimmunocompromised as well as immunocompromised hosts (9). Although one or more putative cellular receptors for CMV have been identified (1, 8, 13, 33), the role of these receptors and the viral components which bind to them in mediating viral infectivity remains unknown.

Herpesviruses infect cells by a series of processes including attachment, fusion of viral envelope with the cell membrane, and transportation of the capsid to the nuclear membrane. The mechanisms by which each stage of infection takes place are unclear. It is thought that herpes simplex virus (HSV) may attach to cells via heparan sulfate moieties on the cell surface (36); the viral component(s) involved in this interaction appear to include gC and may involve other glycoproteins as well (5). At least three glycoproteins (gB, gD, and gH) are probably also involved in penetration of HSV into the cell (2, 4, 6). Epstein-Barr virus binds to CR2 via attachment of gp350 and possibly also gp220 (24, 32, 35), and viral entry then appears to take place via either endocytosis or fusion of virus with the cell membrane, processes which appear to involve gp85 on the viral envelope (22, 23, 25, 31, 32). Varicella-zoster virus gpIII and HSV gH have also been shown to function in cell-cell fusion (syncytium formation) (7, 15), a process which may facilitate direct cell-to-cell spread of virus.

We previously generated anti-idiotype antibodies that mimic gp86 of human CMV and then used those antibodies to identify a 92.5-kDa cell membrane component on human fibroblasts which appears to be a specific receptor for gp86 (13, 14). Previous experiments indicated that preincubation of human embryonic lung (HEL) cells with either of these antibodies resulted in a dose-dependent decrease in CMV infectivity as measured by a standard plaque assay (13). To better understand the role of gp86 and its receptor in CMV infectivity, we attempted to block the early phases of viral infection (viral attachment and virus-cell fusion) with these anti-idiotype antibodies.

The effect of preincubation of HEL cells with either anti-idiotype antibody on CMV attachment was compared with that of preincubation with an immunoglobulin M (IgM) control antibody. HEL cells, maintained and passaged as previously described (14, 28), were grown to confluence in 24-well tissue culture plates (Costar, Pleasanton, Calif.), fixed with 0.1% paraformaldehyde, and blocked with 5% fetal calf serum in phosphate-buffered saline (PBS) at room temperature for 30 min. Cultures were then incubated at 37°C for 1 h with various concentrations of purified monoclonal antibodies (4-3-5 and 6-5-1) which were produced, affinity chromatography purified, and characterized as described elsewhere (13, 14) or with BALB/c IgM control antibody (Southern Biotechnology Associates, Birmingham, Ala.) which was purified in the same manner as the anti-idiotype antibodies. The cultures were then rinsed with Eagle's minimum essential medium and further incubated at 37°C for 1 h with [^{3}H]leucine-labeled, sucrose density-purified CMV (prepared by incubating CMV-infected HEL cells in Eagle's minimum essential medium containing 30% normal concentration of leucine [MEM Select Amine Kit; GIBCO, Long Island, N.Y.] plus 10% fetal calf serum and 1% antibiotic-antimycotic solution for 5 h at 37°C and then adding 50 μCi of ^{3}H-leucine [cell-labeling grade; Amersham, Arlington Heights, Ill.] per ml, harvesting extracellular virus on day 5 of incubation, and purifying radiolabeled virus by sucrose density gradient centrifugation). Alternatively, unfixed confluent HEL cells were blocked with 5% fetal calf serum, incubated at 4°C for 1 h with antibodies, rinsed carefully with Eagle's minimum essential medium, and finally incubated with ^{3}H-labeled CMV at 4°C for 1 h. After final rinses, the acid-precipitable counts in each well were determined.

Low concentrations of control IgM antibody (0.05 to 5 μg per well) did not appreciably affect binding of [^{3}H]leucine-labeled CMV to paraformaldehyde-fixed HEL cells compared with PBS alone (Fig. 1); higher concentrations (50 μg per well) had a slight inhibitory effect on viral binding, presumably due to nonspecific protein binding. Although both anti-idiotype antibodies (4-3-5 and 6-5-1) also did not affect virus binding at low concentrations, they reproducibly enhanced viral binding by 20 to 30% at higher concentrations (Fig. 1). This enhancement was a consistent finding, occur-

* Corresponding author.

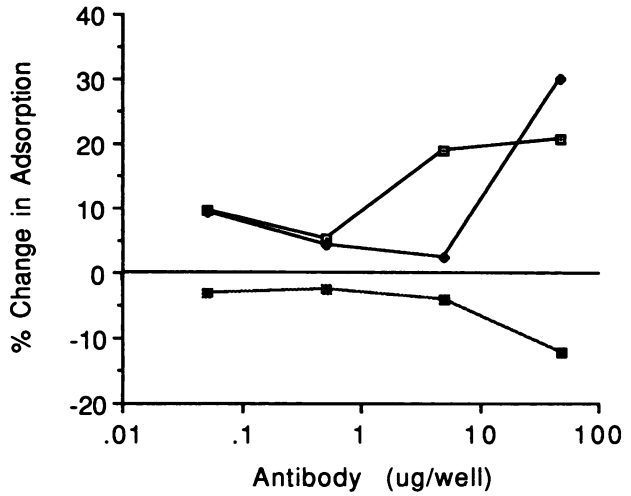

FIG. 1. Effect of monoclonal anti-idiotype antibodies on the binding of ^{3}H-labeled CMV to HEL cells. Fixed HEL cells were preincubated with 4-3-5 (⊡), 6-5-1 (◆), or control IgM antibody (■) prior to incubation with [^{3}H]leucine-labeled CMV strain AD169. Data are expressed as the percent change in viral adsorption relative to adsorption onto HEL cells preincubated with PBS alone.

ing in six experiments. A similar enhancement of virus binding was seen when unfixed cells were preincubated at 4°C with high concentrations of 4-3-5 and 6-5-1, indicating that perhaps the interaction between the anti-idiotype antibodies and the cell surface receptor for gp86 in some way positively influences the binding of CMV to its attachment receptor.

To determine whether gp86 and its receptor function in fusion of CMV with the cell membrane, we used an assay in which a fluorescent amphiphile (octadecyl rhodamine B chloride [R_{18}]) is incorporated into the viral envelope. This assay, originally developed by Hoekstra et al. to study Sendai virus fusion (11), has also been used to study the fusion of Epstein-Barr virus and other viruses (17, 22). We first determined that the insertion of R_{18} into the CMV envelope did not alter its ability to attach to HEL cells. Unlabeled or [^{3}H]leucine-labeled CMV strain AD169 (2×10^{11} PFU) was incubated with 15 nmol of R_{18} (Molecular Probes, Eugene, Oreg.) which was solubilized in 10 μl ethanol for 1 h at room temperature in the dark. R_{18}-labeled virus and unincorporated R_{18} were then separated on a Sephadex G-75 column (Sigma, St. Louis, Mo.), and labeled virus was stored at −70°C. The binding of double-labeled (with [^{3}H]leucine-R_{18}) CMV to HEL cells was then compared with that of CMV labeled with [^{3}H]leucine alone. Both preparations of virus were chromatographed on Sephadex G-75 prior to incubation with fixed HEL cells. When an equivalent amount of counts per minute of each sample was applied to the cells, there was no discernible difference in binding as determined by the acid-precipitable counts recovered.

Further evidence that R_{18} did not affect attachment or other stages of CMV infectivity was provided by plaque titration of R_{18}-labeled virus and unlabeled virus (treated with an equivalent amount of ethanol). Both viral preparations had titers which were essentially equivalent to each other (R_{18}-labeled virus, 2.5×10^{11} PFU/ml; ethanol-treated control virus, 2.4×10^{11} PFU/ml), as well as to that of untreated control virus (2×10^{11} PFU/ml), indicating that neither R_{18} incorporation nor treatment with the small amounts of ethanol required for R_{18} incorporation significantly affected virus infectivity.

The fusion of R_{18}-labeled CMV to HEL cells was then monitored by fluorescence microscopy (Fig. 2). Although a few scattered points of fluorescence emission were evident immediately following addition of R_{18}-labeled CMV (Fig. 2A), the appearance of cell outlines was not evident until approximately 10 min of incubation for those cultures pre-

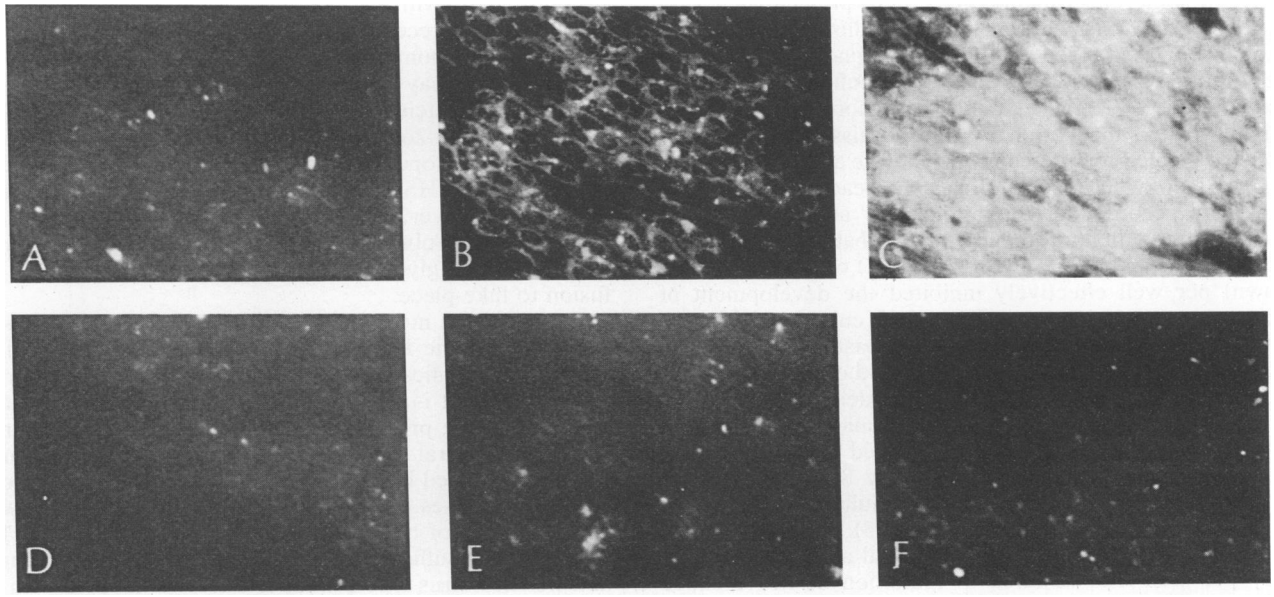

FIG. 2. Effect of 4-3-5 on relief of self-quenching of R_{18}-labeled CMV incubated with HEL cells. HEL cells were preincubated with IgM control antibody (A to C) or 4-3-5 (D to F) and then further incubated with R_{18}-labeled CMV at 4°C. Virus-cell fusion was then allowed to take place by further incubation at 37°C, shown at 0 min (A and D), 10 min (B and E), and 40 min (C and F).

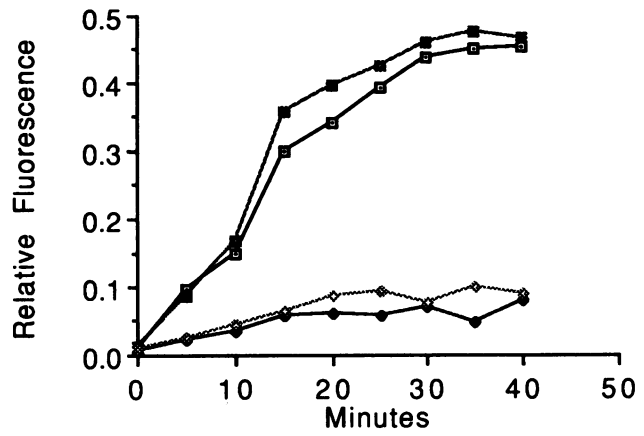

FIG. 3. Quantification of relief of self-quenching of R_{18}-labeled CMV and the effect of preincubation with 4-3-5 or 6-5-1. Relative fluorescence emitted per high-powered field was determined for cultures preincubated with PBS (■), IgM control antibody (⊡), 4-3-5 (◇), or 6-5-1 (◆).

treated with control IgM antibody (Fig. 2A to C) or PBS alone (data not shown). The entire fusion process appeared to be maximal by 40 min of incubation, by which time the outlines of cell processes and nuclei were clearly evident throughout each field (Fig. 2C). When the HEL cells were prefixed with paraformaldehyde, essentially no fluorescence emission beyond that seen at 0 min was evident throughout a 60-min period of incubation, suggesting that membrane fusion did indeed take place to result in fluorescence emission in unfixed cultures.

To determine whether the gp86 receptor might be involved in the process of CMV-HEL cell membrane fusion, we measured fluorescence emission in HEL cells which were preincubated with 0.05 to 50 μg of purified antibody (4-3-5, 6-5-1, or IgM control) per well at 4°C for 1 h followed by 10 μl of R_{18}-labeled CMV per well at 4°C for 30 min. Alternatively, in some experiments, cells were preincubated with R_{18}-labeled CMV at 4°C for 30 min to allow attachment to take place, rinsed twice with cold PBS, and then incubated with antibody at 4°C for 1 h. The cultures were then incubated at 37°C and examined for fluorescence periodically during this incubation by using a Zeiss axioplan microscope connected to a Perceptronics image analyzer (Perceptronics, Knoxville, Tenn.). Images of each high-powered field were recorded at 5-min intervals and subsequently analyzed for mean fluorescence. Preincubation of HEL cells with 50 μg of either 4-3-5 (Fig. 2D to F) or 6-5-1 (data not shown) per well effectively inhibited the development of fluorescence emission seen in control cultures. Although some increase in fluorescence over the baseline was evident in these cultures during the 40-min period of incubation, the outline of cell processes or nuclei evident in the control cultures was never seen. Attempts to quantify the relative amount of fluorescence per high-powered field by using an image analyzer revealed approximately 85% inhibition of fluorescence emission at 40 min as a result of preincubation with either anti-idiotype antibody (Fig. 3). Preincubation of the cells with 5 μg of either monoclonal anti-idiotype antibody per well also resulted in inhibition of fluorescence emission compared with that from preincubation with 5 μg of control antibody per well, although to a lesser extent (approximately 30%); 0.05 or 0.5 μg of antibody per well did not appreciably inhibit fluorescence emission. When virus adsorption was allowed to take place at 4°C prior to incubation with 50 μg of anti-idiotype antibodies per well, inhibition of fusion was still evident but was reduced to approximately 52%.

Because of the possibility that CMV was binding to the Fc portion of cell surface-associated anti-idiotype antibodies and that it thereby could be taken up and inactivated by the cell (rather than inhibited in its fusion with the cell membrane), the fusion experiments were repeated using Fab' and $F(ab')_2$ fragments of 4-3-5, prepared by a modification (16) of a previously described method for trypsin digestion of IgM antibodies (3). Monoclonal antibody (0.5 mg/ml) was buffered in 50 mM Tris (pH 8.1) containing 0.01 M L-cysteine (Sigma); trypsin (Sigma) was then added at a 1:50 ratio of trypsin to IgM, and the mixture was incubated for 6.5 h. The reaction was then stopped with trypsin inhibitor (Sigma), free SH groups were alkylated with iodoacetamide, and the mixture was dialyzed in PBS overnight at 4°C. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed no detectable whole antibody, approximately 67% Fab' fragments, and 33% $F(ab')_2$ fragments in this preparation. Fluorescence emission from R_{18}-labeled-CMV-infected HEL cells preincubated with Fab' and $F(ab')_2$ fragments from 50 μg of 4-3-5 per well was decreased by approximately 75% compared with the control IgM antibody value after 40 min of incubation.

Our results indicate that neither gp86 nor its 92.5-kDa fibroblast membrane receptor are primary mediators of human CMV attachment to HEL cells. This finding is consistent with recent reports which identify CMV adsorption receptors of much lower molecular weights. For example, a cell membrane receptor for radiolabeled virions has been identified on T cells and fibroblasts which has a molecular weight of approximately 30,000 to 34,000 (1, 33), and earlier data indicated that other lower-molecular-weight molecules such as human leukocyte antigen determinants may also serve as cell membrane receptors for β-2 microglobulin-CMV complexes (8). These data suggest that gp86 functions as a fusion protein of human CMV, facilitating fusion of virus with the cell membrane. This hypothesis is consistent with recent reports which indicate that glycoproteins that have homology to gp86 from certain other human herpesviruses may facilitate fusion of virus with the cell membrane (Epstein-Barr virus) (22), function in cell-cell fusion (varicella-zoster virus and HSV) (7, 15), or, along with other envelope glycoproteins (gB and gD), allow penetration of virus (HSV) (6, 7) into the cell. It is not clear from our data, however, whether gp86 is the only human CMV glycoprotein involved in the process of virus-cell fusion or whether other glycoproteins may also be necessary for fusion to take place.

Because both monoclonal antibodies which block fusion of CMV with the HEL cell membrane and affinity-purified gp86 bind specifically to a 92.5-kDa HEL cell membrane receptor (13), it is conceivable that the gp86 receptor also functions in the process of virus-cell fusion. In addition to receptors for viral adsorption, other cell membrane components may indeed be necessary for the penetration of some enveloped viruses. For example, although CD4 is necessary for adsorption of human immunodeficiency virus type 1 to cells, it is not sufficient for viral penetration; human immunodeficiency virus type 1 binds to but does not fuse with the membranes of mouse cell lines which express CD4, and the virus therefore does not penetrate these cells (12, 18). Similarly, when cells are preincubated with peptides that

correspond to the N-terminal hydrophobic domain of specific fusion glycoproteins from the envelopes of either Sendai virus or measles virus, viral infectivity and syncytium formation are inhibited, suggesting that specific receptors for the fusion proteins of these viruses may be present on the cell surface (12, 21, 27, 29, 30, 34). Further evidence for such receptors is provided by the fact that only one of six Sendai virions that bind to erythrocyte cell membranes will fuse with the membranes, even though all virions have the capacity to fuse with these cells (10, 19, 26).

Our data suggest that the previously identified receptor for gp86 may indeed be such a fusion protein receptor for human CMV which may be necessary for viral penetration. The fact that the anti-idiotype antibodies were not able to inhibit fusion as well when virus was preadsorbed to the cells is consistent with the hypothesis that both gp86 and antibodies bind to the same fusion protein receptor or that preadsorbed virus sterically or otherwise nonspecifically interfered with subsequent binding of the antibodies to another cell membrane component that affects virus-cell fusion. Ultimately, experiments in which the gp86 receptor is incorporated into the membrane of cells which have an adsorption receptor but lack the fusion receptor (as used to demonstrate the function of a human poliovirus receptor by transformation of its gene into mouse cells [20]) may be necessary to definitively determine its function.

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