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Short Communication

# Selective cytotoxicity towards cytomegalovirus-infected cells by immunotoxins consisting of gelonin linked to anti-cytomegalovirus antibody

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**Abstract**

An immunotoxin specific for cells infected with human cytomegalovirus (HCMV) was constructed by attaching the ribosome-inactivating enzyme, gelonin, through a disulfide linkage to polyclonal human immunoglobulin (IgG). In uninfected cells, there was no difference between [$^{35}$S]methionine incorporation in untreated cultures and those treated with immunotoxin at 100 $\mu$g/ml. In HCMV-infected cells, there was a significant decrease in [$^{35}$S]methionine incorporation in the immunotoxin-treated cultures, suggesting a selective cytotoxic effect on the virus-infected cells. An immunotoxin specific for murine cytomegalovirus (MCMV) was prepared by linking gelonin to polyclonal anti-MCMV IgG. Using this same parameter for assay of cytotoxicity, the anti-MCMV immunotoxin had a 50% cytotoxic concentration of 35 $\mu$g/ml in MCMV-infected cells and greater than 200 $\mu$g/ml in uninfected cells. MCMV yields measured at 7 days postinoculation were reduced by 2 $\log_{10}$ in cultures treated with immunotoxin at 20 $\mu$g/ml at 1 day postinoculation. These data suggest immunotoxins may have potential for eliminating CMV-infected cells from the host.

*Keywords:* Immunotoxin; Gelonin; Immunodeficiency; Cytomegalovirus

Drugs for the treatment of cytomegalovirus (CMV) infections in man currently include ganciclovir (DHPG) and phosphonoformate (PFA) (Laskin et al., 1987; Walms-

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ley et al., 1988). Treatment with these drugs strongly inhibits CMV replication at therapeutically acceptable drug concentrations; however, in the immunosuppressed patient lacking an effective cytotoxic immune response, infected cells persist and CMV disease recrudesces upon termination of drug therapy (Laughlin et al., 1991).

An additional component which might be beneficial in CMV therapy in immunodeficient patients is the use of selective cytotoxic agents to eliminate the CMV-infected cells. Immunotoxins are targeted cytotoxic agents which may have potential to accomplish this purpose; they have been applied on an experimental basis for killing cancer cells (Vitetta and Uhr, 1985; Dillman, 1989). Clinical trials with immunotoxins in cancer patients have shown antitumor effects and satisfactory patient tolerance (Laurent et al., 1986; Dillman, 1989; Vitetta et al., 1991; LeMaistre et al., 1992). CMV-specific immunotoxins used in combination with virustatic drugs may eliminate persisting CMV-infected cells and hence prevent the disease recrudescence. The development of anti-CMV immunotoxins will facilitate testing of combination therapies consisting of antiviral drugs targeted at key viral enzymes and immunotoxins targeted to viral antigens displayed on the surface of infected cells. Evaluations of this multicomponent therapeutic approach will require both cell culture and animal studies. Since suitable animal models are not available for human CMV (HCMV), these studies may appropriately use the murine virus (MCMV) for both in vitro and animal experiments, and HCMV for in vitro experiments to demonstrate that efficacy seen with one virus can be repeated with the other.

This report describes initial studies on the selective cytotoxic activity of anti-CMV immunotoxins constructed by linking polyclonal anti-HCMV and MCMV IgG to gelonin, a 30-kDa single-chain ribosome-inactivating enzyme which has no inherent ability to enter cells and thus very little inherent cytotoxicity (Stirpe et al., 1980).

The gelonin used was obtained from Pierce Chemical Co. (Rockford, IL). Polyclonal IgG for use in the MCMV–gelonin immunotoxin was isolated by protein A affinity chromatography from the sera of rabbits hyperimmunized to MCMV. The HCMV–gelonin immunotoxin (CytG–gelonin) was constructed using similarly purified polyclonal human IgG isolated from CytoGam™, HCMV immune globulin (Connaught Laboratories, Inc., Swiftwater, PA). The IgGs were conjugated to gelonin through a disulfide linkage resulting in immunotoxins consisting of one molecule of gelonin attached to one molecule of IgG. The conjugation, purification and characterization procedures for preparing the immunotoxins were previously described (Barnett et al., 1991).

Sellers et al. (1994) described a cell proliferation assay for measuring surviving cell populations after treatment with immunotoxins. They found that the neither the Trypan blue exclusion assay nor a fluorescent dye incorporation assay in any way reflected the killing of cells by ricin, a ribosome-inactivating agent used in a manner similar to gelonin in immunotoxin construction. Both assays greatly underestimated the toxicity of immunotoxins, presumably because the immunotoxin-mediated process of cell death is gradual (Sellers et al., 1994). In the studies described in this report, the cytotoxic effects of MCMV–gelonin were determined on the MCMV-sensitive mouse mammary tumor cell line, C127I (Smee et al., 1989; obtained from American type Culture Collection, ATCC) growing in 96-well culture plates. With the in vitro systems used in these

studies, neither C127I cells nor the MRC-5 cells replicated after they were infected with CMV, for that reason, we were unable to use a cell proliferation assay for measuring cytotoxicity in CMV-infected cells. Inhibition of [$^{35}$S]methionine incorporation, an assay closely linked to the primary mechanism of action of immunotoxins, has been shown to be an accurate measure of the cytotoxic effect of immunotoxins (Pincus and McClure, 1993).

Varying concentrations of immunotoxins were added to uninfected and MCMV-infected (Smith strain of MCMV, obtained from ATCC) cultures of C127I cells 24 h postinoculation (p.i.). In all targeted cytotoxicity studies with either HCMV or MCMV described in this report, a multiplicity of infection (m.o.i.) of 10 $CCID_{50}$/cell was used in an effort to obtain cultures wherein all cells were infected. Immunofluorescent antibody staining of cultures inoculated at an m.o.i. of 10 $CCID_{50}$/cell revealed that more than 95% of the cells expressed CMV antigens by 24 h p.i. There was selective toxicity towards infected cells when cytotoxicity was measured by tetrazolium dye conversion (Green et al., 1984) or by the more precisely quantified activity parameter, [$^{35}$S]methionine incorporation. In the latter method, the medium on the immunotoxin-treated and untreated cells was removed 17 h after immunotoxin was added and replaced with methionine-free medium containing [$^{35}$S]methionine (Tran$^{35}$S-label, ICN Biomedicals Inc., Irvine, CA, spec. act. 43.7 TBq/mmol) at 0.5 $\mu$Ci per well (Pincus and McClure, 1993) and cultures were incubated at 37°C for 4–16 h. Using 0.5 $\mu$Ci per well in a 96-well plate, the amount of $^{35}$S incorporated increased linearly over this time range. A 4-h incorporation period resulted in approximately 50,000 cpm/well incorporated in C127I cell cultures, while an 8-h incorporation period was required with MRC-5 cells to obtain 27,000 cpm/well. Following the $^{35}$S-incorporation period, cells were lysed with water and proteins were precipitated with 10% trichloroacetic acid (TCA). TCA precipitable $^{35}$S was harvested from the cultures onto filter paper and quantified using a FilterMate Harvester and Matrix 9600 Direct Beta Counter, respectively (Packard Instruments Co., Meriden, CT). Data were expressed as percent [$^{35}$S]methionine incorporated compared to untreated controls.

The dose–response of MCMV-infected and uninfected C127I cells to the anti-MCMV immunotoxin is shown in Fig. 1. Inhibition of [$^{35}$S]methionine incorporation was the parameter used to measure cytotoxicity. The 50% inhibitory concentration on infected cells was 36 $\mu$g/ml; there was insignificant toxicity towards uninfected cells at 200 $\mu$g/ml, the highest concentration evaluated. Treatments with the anti-HCMV immunotoxin, CytG–gelonin at 100 $\mu$g/ml, and an unconjugated mixture of anti-MCMV IgG (200 $\mu$g/ml) and gelonin (100 $\mu$g/ml) were included as controls. Neither the CytG–gelonin nor the unconjugated mixture of anti-MCMV IgG and gelonin were cytotoxic to either MCMV-infected or uninfected C127I cells.

The effect of MCMV–gelonin upon virus yield was examined using confluent C127I cell monolayers in 24-well plates inoculated with MCMV at an m.o.i. of 0.001 $CCID_{50}$/cell. At 24 h p.i., the maintenance medium was replaced with medium containing MCMV–gelonin at varying concentrations. Immunotoxin was left on cultures throughout the remainder of infection. At 7 days p.i., when virus yield was maximal in untreated cultures, the cultures were frozen. Both intra- and extracellular virus (combined) were harvested and virus yields (duplicate assays on each of 3 cultures for each

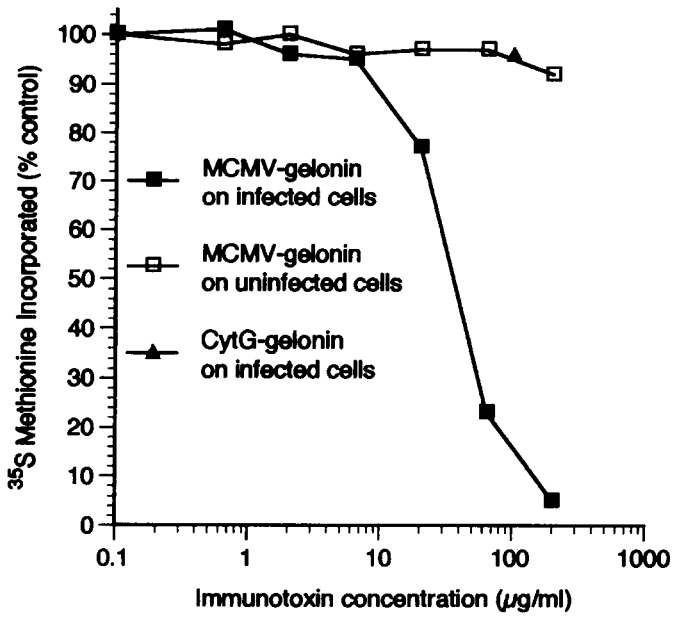


Fig. 1. Cytotoxicity dose–response of immunotoxin MCMV–gelonin on MCMV-infected and uninfected cells. Ninety-six-well plate cultures of C127I cells were inoculated with MCMV at an m.o.i. of 10 $CCID_{50}$/cell. Varying concentrations of immunotoxin were added at 24 h p.i. (16 replicate wells/concentration) and cultures were incubated for 17 h. After the medium was removed, cultures were pulsed with [$^{35}$S]methionine and harvested 4 h later. Among the 3 replicate experiments, slight variations in the amount of labeled methionine added and the length of the incorporation period, resulted in variations in the incorporation baselines, but not in the relative incorporation. The results are [$^{35}$S]methionine incorporation expressed as percent of incorporation in untreated virus-infected or uninfected cells. CytG–gelonin immunotoxin, constructed identically except with human IgG with no specificity for MCMV or C127I cell antigens, showed no activity against MCMV-infected C127I cells at 100 $\mu$g/ml (single triangle). Representative raw count data: in the absence of immunotoxin, the incorporation of [$^{35}$S]methionine in uninfected cells was $54{,}160 \pm 4370$ cpm (mean $\pm$ S.D.).

treatment condition) were determined. Virus was assayed by endpoint dilution on 96-well cultures of C127I cells (Smee et al., 1992). The virus was detected by observing for virus-induced cytopathic effect 7 days after inoculation. Virus yields ($\log_{10}$ $CCID_{50}$/ml $\pm$ S.D.) from cultures containing MCMV–gelonin at 0, 2 and 20 $\mu$g/ml were $7.4 \pm 0.3$, $7.6 \pm 0.5$ and $5.8 \pm 0.5$, respectively. At 200 $\mu$g/ml, toxicity towards uninfected cells was observed after immunotoxin was present for 6 days, although toxicity was not noted in the treatments of 1 day duration as described in Fig. 1. No cytotoxicity towards uninfected cells was evident throughout the 6-day treatment period with immunotoxin at 2 or 20 $\mu$g/ml. The virus yield reduction at 20 $\mu$g/ml was significant ($P = 0.009$, unpaired $t$-test, two-tailed). However, considering the toxicity at 200 $\mu$g/ml, studies utilizing more sensitive procedures for detecting cytotoxicity in

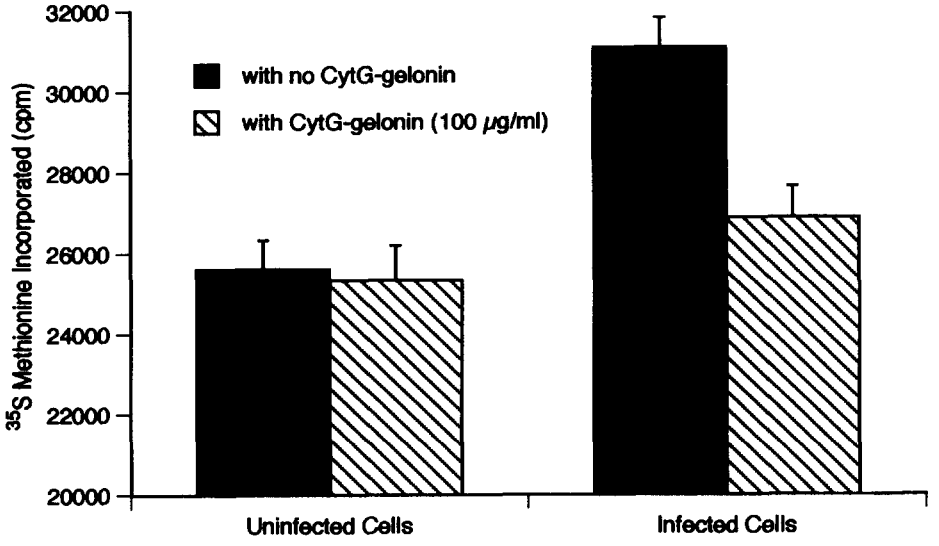


Fig. 2. Cytotoxicity of anti-HCMV immunotoxin CytG–gelonin towards uninfected and HCMV-infected MRC-5 cells. Cells were inoculated at an m.o.i. of 10 $CCID_{50}$/cell and varying dilutions of immunotoxin were added at 24 h p.i. Forty-one hours after the addition of the immunotoxin, the cultures were pulsed with [$^{35}$S]methionine for 8 h. In the absence of immunotoxin, [$^{35}$S]methionine incorporation in uninfected cells was 25,600 cpm and in HCMV-infected cells was 31,060 cpm. The experiment was replicated twice, using 12 replicate wells for each immunotoxin concentration in each experiment. Only at the highest concentration of immunotoxin evaluated, 100 μg/ml, was targeted cytotoxicity observed. The bar graph shows the means + S.E.M. for [$^{35}$S]methionine incorporation in uninfected and HCMV-infected cells with immunotoxin at 100 μg/ml and without immunotoxin. The difference between treated and untreated uninfected cells was not significant ($P = 0.81$), but in infected cells the difference was extremely significant ($P = 0.001$). Controls consisting of the immunotoxin MCMV–gelonin (100 μg/ml) and an unconjugated mixture of CytoGam IgG (200 μg/ml) and gelonin (100 μg/ml) were not significantly cytotoxic to either HCMV-infected or uninfected MRC-5 cells.

non-infected cells upon prolonged exposure to immunotoxin might reveal non-specific toxicity at 20 μg/ml.

CytG–gelonin was evaluated in dose–response studies, against the human diploid cell line, MRC-5, infected with the Towne strain of HCMV at an m.o.i. of 10 $CCID_{50}$/cell (MRC-5 cells and HCMV both obtained from ATCC). This anti-HCMV immunotoxin, although not cytotoxic to MCMV-infected cells, was found significantly cytotoxic ($P = 0.001$, $t$-test) at 100 μg/ml (the highest concentration that we were able to evaluate) to HCMV-infected MRC-5 cells, but not uninfected cells (Fig. 2). Lower concentrations of CytG–gelonin were not significantly cytotoxic to either infected or uninfected cells. Controls similar to those included in the MCMV toxicity evaluations showed that neither MCMV–gelonin at 100 μg/ml nor an unconjugated mixture of CytoGam IgG (200 μg/ml) and gelonin (100 μg/ml) were cytotoxic to either HCMV-infected or uninfected MRC-5 cells. It was noted that the [$^{35}$S]methionine incorporation was overall higher in the infected cells than in the uninfected cells at the

time of assay. This was presumably a result of HCMV stimulation of host cell macromolecular biosynthesis (Stinski, 1977).

The anti-HCMV and anti-MCMV immunotoxins described in these studies were constructed from polyclonal hyperimmune IgG. The virus specific antibody in these preparations accounted for less than a few percent of the total IgG. The lower cytotoxicity of the CytG–gelonin, in comparison to the MCMV–gelonin, may have resulted from a lower proportion of anti-CMV specific IgG in the pooled human IgG or might relate to more fundamental differences in the cell lines, viruses or level of CMV-antigen expression in the two cell culture systems.

In the future, combination therapy with a known CMV inhibitor and immunotoxin will be evaluated. DHPG strongly inhibits CMV replication and could be predicted to interfere with anti-CMV immunotoxin activity by blocking the expression of CMV antigens on infected cells. Yet since DHPG and other anti-CMV antivirals generally act at the viral DNA replication level, it is not surprising that CMV proteins are synthesized in infected cells, even in the presence of drug (Mar et al., 1982, 1983). CMV-specific immunofluorescent antibody staining revealed CMV antigens at 24 h p.i. in both MCMV-infected and HCMV-infected cultures with or without DHPG present at 10 $\mu$g/ml beginning 1.5 h p.i. In the same cytotoxicity studies described above, DHPG (10 $\mu$g/ml) added at 1.5 h p.i. had no effect on immunotoxin cytotoxicity in either infected or uninfected cultures, indicating that the targeted intoxication proceeded via CMV antigens expressed even in the presence of therapeutic levels of DHPG. DHPG at 10 $\mu$g/ml is extremely inhibitory to MCMV and HCMV replication (Smee et al., 1983). It is possible that immunotoxins constructed with appropriate anti-CMV monoclonal antibodies in place of the polyclonal antibodies used in the present study will have increased specific activity. One of the challenges of identifying such monoclonal antibodies that will be effective as the targeting portion of an immunotoxin in the proposed combination therapies will be the selection of monoclonal antibodies towards CMV antigens that are expressed on the surface of infected cells while the CMV infection is being inhibited by DHPG.

Both MCMV- and HCMV-infected cells are susceptible to the corresponding CMV-specific immunotoxins, a result similar to what we have previously reported with immunotoxins constructed against an arenavirus (Barnett et al., 1991). The data indicate the immunotoxins are selectively cytotoxic to CMV-infected cells, and thus might eliminate CMV-infected cells from the host. It will be important to determine if such immunotoxins will also be effective in animals infected with MCMV; studies to determine this are currently underway. Others have reported on immunotoxins active against HIV in cell culture (Chaudhary et al., 1988; Till et al., 1988; Pincus et al., 1989; Matsushita et al., 1990).

The acceptance of immunotoxins as therapeutic tools will depend, in part, upon the ability of the host to tolerate these agents. Immunotoxins constructed with deglycosylated ricin A chain have been found to be quite well tolerated in man (Vitetta et al., 1991); we are now utilizing this toxin and monoclonal antibodies in producing additional immunotoxins directed at CMV.

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