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Short communication

Combination of antiviral immunotoxin and ganciclovir or cidofovir for the treatment of murine cytomegalovirus infections

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Abstract

The effects of two anti-murine cytomegalovirus (MCMV) immunotoxins used in combination with ganciclovir (GCV) or cidofovir (HPMPC) against MCMV were determined in vitro and in mice. The inhibitors were added to cell cultures 24 or 48 h after MCMV adsorption so as to not affect the initial infection rate. The immunotoxins (0.63, 1.25 and 2.5 μg/ml) combined with GCV (1.25, 2.5 and 5 μM) or HPMPC (0.03, 0.06 and 0.12 μM) caused synergistic inhibition of virus yield in C127I cells at most of the combinations tested. No toxic effect on cell growth in culture was observed at these immunotoxin/drug combinations. The effects of immunotoxin and GCV treatment were studied further in MCMV-infected severe combined immunodeficient (SCID) mice. Immunotoxin (1 mg/kg per day) given by intraperitoneal (i.p.) injection on days 1, 4 and 7 of the infection did not extend the mean day to death compared with the placebo group. Once daily i.p. treatment with GCV (50 mg/kg per day) for 7 days starting at 24 h after virus inoculation extended survival time almost 11 days. The combination of immunotoxin plus GCV was better than GCV alone, extending the mean day to death an additional 2 to 3 days, which is suggestive of a synergistic effect.

Keywords: Immunotoxin; Ganciclovir; Cidofovir; SCID mice; Cytomegalovirus; Antiviral

Human cytomegalovirus (HCMV) causes serious infections in immunocompromised individuals (Yow, 1989; Rubin, 1990; Schooley, 1990). The incidence of serious infections caused by this virus has continued to increase with the expansion of the AIDS epidemic. Antiviral drugs approved to date for the treatment of cytomegalovirus infections include ganciclovir (GCV) (Laskin et al., 1987), foscarnet (Walmsley et al., 1988) and cidofovir (HPMPC) (Lalezari et al., 1995; Polis et al., 1995). All of these substances exhibit dose-limit-

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ing toxic side effects in patients. Hyperimmune globulin derived from pooled sera from seropositive individuals is being used in organ transplant recipients as a prophylactic measure (Emanuel, 1991). Many clinicians use a combination of antiviral agent plus hyperimmune globulin in their transplant patients (Tenschert et al., 1993).

Antibody-based immunotoxins are polyclonal or monoclonal antibodies linked to toxins (such as gelonin or ricin A chain) which have the potential to bind to and kill virus-infected cells (Barnett et al., 1991; Pincus et al., 1989). We recently reported that gelonin and ricin A chain immunotoxins were effective against murine cytomegalovirus (MCMV) in vitro (Barnett et al., 1995, 1996). The gelonin immunotoxin was prepared with polyclonal anti-MCMV rabbit serum whereas the ricin A chain immunotoxins were constructed using two different mouse monoclonal antibodies. In those reports, treatment of MCMV-infected cells for 1 day with the immunotoxins resulted in dose-responsive inhibition of protein synthesis, indicative of immunotoxin activity. In the present studies, we extend the observations further to evaluate the effects of continuous treatment with the ricin A chain immunotoxins on MCMV yield from infected cells and on cytotoxicity when combined with GCV or HPMPC.

For cell culture studies, the Smith strain of MCMV and C127I mouse mammary tumor cells, obtained from the American Type Culture Collection, Rockville, MD, were used. Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was the cell growth medium. During experiments the serum concentration was reduced to 2%. A cell-culture-passaged virus was prepared for in vitro experiments and a mouse-passaged virus was used for animal studies (Freitas et al., 1985). Although the cell-culture-passaged and mouse-passaged viruses replicate similarly in vitro, the latter virus is more virulent in mice (Selgrade et al., 1981).

Ganciclovir (GCV, Syntex, Palo Alto, CA) was purchased from a local pharmacy. (*S*)-1-[3-Hydroxy-(2-phosphonylmethoxy)propyl]cytosine (cidofovir, HPMPC) was kindly provided by Dr Norbert Bischofberger, Gilead Sciences, Foster City, CA. The compounds were dissolved in sterile cell culture medium for in vitro work or in sterile saline for animal studies.

Two IgG_{2a} subclass monoclonal antibodies against MCMV (C34.18.F6 and D5.F10.B8) coupled to deglycosylated ricin A chain, designated C34-dgA and D5-dgA (Smee et al., 1995), were used to prepare immunotoxins. The uncoupled C34.18.F6 antibody by itself was found not to neutralize MCMV whereas the D5.F10.B8 antibody did. The D5.F10.B8 antibody was previously shown to inhibit MCMV either alone or combined with GCV or HPMPC (Smee et al., 1995).

Virus yield reduction assays of combined intracellular and extracellular virus were conducted with confluent monolayers of C127I cells in 24-well plates (Smee et al., 1995) which were infected with 320 plaque forming units (PFU) of MCMV per well. A low multiplicity of infection (MOI) was used in these studies to mimic the in vivo infection which was initiated with a low virus inoculum. Previously we showed immunotoxic activity under high MOI conditions (Barnett et al., 1996). On day 1 after infection, the immunotoxin and/or drug was applied. At 7–8 days, when cytopathology in immunotoxin- and drug-free wells was 100%, the cells and supernatant fluids were collected, sonicated, and stored frozen at −80°C. To titrate for virus yield, 96-well plate cultures of C127I cells were exposed to varying dilutions (made with a ProPette, Perkin-Elmer, Emeryville, CA) of virus-containing samples for a week. Virus titers were determined by end point dilution method (Reed and Muench, 1938) and expressed as $\log_{10}$ 50% cell culture infectious doses per ml ($\log_{10}$ $CCID_{50}$/ml). The combined results of two separate experiments are presented in the Tables. Mathematical calculations of drug interactions shown in the Tables were made by the methods described by Schinazi et al. (1982). Results of this method of assessing synergy were confirmed using the three-dimensional method of Pritchard and Shipman (1990). For the sake of brevity, three-dimensional synergy plots are not presented in this report.

Virus yield reduction was chosen over other assay methods (such as inhibition of cytopathic

effect, plaque reduction, or inhibition of viral antigen production) for studying the effects of the inhibitors. This is the most direct assay for actually quantifying the amount of infectious virus produced.

In order to study the effects of the immunotoxins and drugs, alone or in combination, on the proliferation of uninfected cells, 2×10^4 C127I cells were seeded into each well of 24-well plates. After 24 h, when cells were attached to the plates and beginning to replicate, varying concentrations of immunotoxin and/or drug were added to the cultures. After 4 days, the cells were enumerated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described previously (Smee et al., 1992a). This required transfer of 100 μl of the MTT formazen product from each well to a 96-well plate in order to be quantified by an enzyme-linked immunosorbent assay (ELISA) plate reader measuring optical density at 600 nm.

Initially, for cell culture experiments, the proper concentrations of immunotoxins needed to be identified. The concentrations desired were those which were not completely inhibitory to virus replication and yet were also non-toxic to cells. Treatment of MCMV-infected cells with each immunotoxin at 1.25–20 μg/ml caused a 2–5 $\log_{10}$ decrease in virus titer (data not shown). No antiviral activity was present at 0.63 μg/ml. Cell proliferation was also inhibited at C34-dgA immunotoxin concentrations of 5 μg/ml and above or D5-dgA immunotoxin concentrations of over 10 μg/ml. No effect on cell proliferation was evident at 2.5 μg/ml for either immunotoxin. Thus, for later antiviral drug combination studies, the immunotoxins were evaluated at 0.63, 1.25 and 2.5 μg/ml to avoid cytotoxicity.

By itself, GCV was active in suppressing MCMV titers at 1.25, 2.5 and 5 μM, whether applied at 24 or 48 h after virus inoculation (Table 1). D5-dgA immunotoxin was active by itself in this experiment at 1.25 and 2.5 μg/ml, whereas C34-dgA immunotoxin inhibited virus production by itself only at 2.5 μg/ml. When GCV was combined with either of the immunotoxins, synergistic inhibition of virus occurred at most of the drug combinations tested, whether treatment started at 24 or 48 h after virus inoculation. Concentrations of immunotoxins previously inactive by themselves were effective when combined with GCV.

HPMPC was effective in reducing MCMV yield in a dose-responsive manner at concentrations of 0.03, 0.06 and 0.12 μM (Table 2). When HPMPC was combined with immunotoxins, synergistic interactions were apparent using 0.63, 1.25 and 2.5 μg/ml of the immunotoxins. Results at 48 h were similar to those obtained when the compounds were applied at 24 h in terms of the combined antiviral effect. Concentrations of these immunotoxins previously inactive by themselves were effective when combined with HPMPC.

The immunotoxins and GCV or HPMPC were evaluated in combination for toxicity in rapidly growing uninfected C127I cells, using the concentrations reported in Tables 1 and 2. After 4 days of proliferation, none of the drug/immunotoxin combinations inhibited the replication of these cells, indicating a lack of toxicity at the concentrations that showed antiviral synergy (data not shown).

For animal experiments, severe combined immunodeficient (SCID) mice were raised under aseptic conditions at the Utah State University Laboratory Animal Research Center, Logan, UT. They were infected i.p. with MCMV at a dose of 2×10^4 PFU per mouse, which generally causes death in 15–20 days. Immunotoxin treatments were given i.p. once daily on days 1, 4 and 7 after virus challenge. GCV was administered i.p. once daily for 7 days starting 1 day after virus inoculation. The short-term nature of these treatments did not result in overt toxicity to the mice. There were 7 mice in each treatment group. Deaths were recorded daily for 35 days. Extensions in mean day to death were statistically analyzed by the two-tailed Mann–Whitney *U*-test. In this model it was not possible to prevent mortality by short-term antiviral drug treatment (Smee et al., 1992b), although life span could be extended considerably.

The potential of immunotoxin to interact synergistically with GCV was evaluated in MCMV-infected SCID mice (Table 3). Immunotoxins by themselves did not increase mean survival time

Table 1
Effects of D5-dgA and C34-dgA immunotoxins combined with GCV on MCMV yields from C127I cells

GCV conc. (μM)	Virus yield[a] ($\log_{10}$ $CCID_{50}$/ml) in the presence of:						
	No immunotoxin	D5-dgA (0.63 μg/ml)	D5-dgA (1.25 μg/ml)	D5-dgA (2.5 μg/ml)	C34-dgA (0.63 μg/ml)	C34-dgA (1.25 μg/ml)	C34-dgA (2.5 μg/ml)
Drug and immunotoxin applied 24 h after virus inoculation							
0	$\geq$7.0	$\geq$7.0	6.3	4.3	$\geq$7.0	$\geq$7.0	5.7
1.25	5.7	*3.0*[b]	*3.3*	*2.0*	*3.0*	*3.7*	*3.0*
2.5	3.0	2.5	2.7	*1.5*	3.3	2.7	2.5
5.0	2.3	*1.5*	*1.3*	1.5	*1.7*	*1.7*	*1.3*
Drug and immunotoxin applied 48 h after virus inoculation							
0	$\geq$7.0	$\geq$7.0	6.0	4.0	$\geq$7.0	$\geq$7.0	5.0
1.25	6.5	*4.0*	*3.3*	*2.3*	*5.3*	*3.7*	*2.7*
2.5	4.7	3.5	*2.7*	*2.0*	4.5	*3.3*	3.0
5.0	4.0	*2.0*	*2.0*	*< 1.0*	*2.7*	*2.0*	*1.7*

[a] GCV and immunotoxin were applied to MCMV-infected cells (MOI = 0.01 virus/cell) in 24-well plates. Virus yields (combined intra- and extracellular virus) were determined after 6 days of virus replication.
[b] Italicized values indicate synergistic inhibition of virus yield.

Table 2
Effects of D5-dgA and C34-dgA immunotoxins combined with HPMPC on MCMV yields from C1271 cells

HPMPC conc. (μM)	Virus yield[a] ($\log_{10}$ $CCID_{50}$/ml) in the presence of:						
	No Immunotoxin	D5-dgA (0.63 μg/ml)	D5-dgA (1.25 μg/ml)	D5-dgA (2.5 μg/ml)	C34-dgA (0.63 μg/ml)	C34-dgA (1.25 μg/ml)	C34-dgA (2.5 μg/ml)
Drug and immunotoxin applied 24 h after virus inoculation							
0	⩾7.0	⩾7.0	6.3	4.3	⩾7.0	⩾7.0	5.7
0.03	5.0	*3.7*[b]	*3.7*	4.0	*4.3*	*3.5*	*3.0*
0.06	4.0	*2.7*	*2.5*	*1.5*	*2.7*	*2.7*	*2.3*
0.12	3.5	*2.3*	*1.7*	*1.5*	*2.7*	*2.7*	*2.3*
Drug and immunotoxin applied 48 h after virus inoculation							
0	⩾7.0	⩾7.0	6.0	4.0	⩾7.0	⩾7.0	5.0
0.03	6.5	*3.7*	*2.0*	*2.7*	*5.0*	*3.3*	*3.0*
0.06	5.0	*3.3*	*2.3*	*2.3*	*3.3*	*3.0*	*2.5*
0.12	3.3	*2.5*	*1.7*	1.7	3.5	3.3	2.3

[a] HPMPC and immunotoxin were applied to MCMV-infected cells (MOI = 0.01 virus/cell) in 24-well plates. Virus yields (combined intra- and extracellular virus) were determined after 6 days of virus replication.
[b] Italicized values indicate synergistic inhibition of virus yield.

relative to the placebo control. GCV alone at 50 mg/kg per day increased survival time significantly by 11 days. The mean day to death in the immunotoxin/GCV groups was 2–3 days longer than in the GCV group, indicating a moderate improvement in survival. In the SCID mouse model, a 3-day shift in mean day to death correlates to approximately a one $\log_{10}$ decrease in virus titer (Smee et al., 1992b). We were limited in the amount of available immunotoxin for these studies, so did not quantify tissue virus titers in other mice that would have been required for this purpose. The overall effect of the combination treatments was suggestive of synergy when analyzed by the method of Schinazi et al. (1982).

These results demonstrate that immunotoxins combined with GCV or HPMPC produced a synergistic inhibition of MCMV yield in cell culture. The effect of immunotoxins combined with GCV in MCMV-infected SCID mice appeared to be only suggestive of synergy, however. The immunotoxins were not sufficiently active in vivo to cause a more profound effect on survival. The reasons for the disparity between cell culture and animal results may relate to pharmacokinetics of the immunotoxins in mice. The amount of immunotoxin given to each animal was 20 μg per mouse or 1.0 mg/kg administered intraperitoneally. Since the blood volume of this size of mouse is about 2 ml, the maximum serum level of antibody was estimated to be 10 μg/ml, which would have been adequate for a potent antiviral effect in the in vitro studies. Actual serum levels achieved were not determined, and how long the highest concentration of immunotoxin persisted in vivo is uncertain. The immunotoxins may not have been able to reach or persist at the sites of virus replication in sufficient concentration, based upon the fact that immunotoxins alone did not prolong the life of infected mice.

One purpose of giving drugs in combination is to improve treatment efficacy, which was accomplished to a moderate degree in the in vivo experiment. Another purpose is to reduce host toxicity. Cell proliferation experiments established that GCV plus immunotoxin in various combinations did not result in increased cytotoxicity over the range of concentrations tested. Overall, the relative effect of immunotoxins was weak in comparison to the effect of GCV in mice. Further work will need to be conducted to determine if more potent and effective immunotoxins against MCMV can be found, the results of which may help understand the role of immunotoxins in the treatment of human cytomegalovirus infections.

Table 3
Treatment of MCMV-infected SCID mice with D5-dgA or C34-dgA immunotoxin and GCV alone or in combination

Treatment[a] (mg/kg per day)	Mean day to death
Placebo	16.3 ± 1.4
GCV (50)	27.1 ± 2.5*
C34-dgA (1)	16.6 ± 2.0
D5-dgA (1)	16.0 ± 2.1
GCV (50) + C34-dgA (1)	29.3 ± 1.1*
GCV (50) + D5-dgA (1)	30.4 ± 3.1*

[a] GCV was given i.p. once daily for 7 days starting 24 h after virus inoculation. Immunotoxins were administered i.p. on days 1, 4 and 7 of the infection.

* Statistically significant ($P < 0.001$) difference between this group and the placebo control. Survival of the GCV group was not significantly different from the groups treated with immunotoxin combined with GCV.

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