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# Antiviral immunotoxins: antibody-mediated delivery of gelonin inhibits Pichinde virus replication in vitro

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## Summary

Immunotoxins were produced and evaluated for antiviral activity against Pichinde virus, a member of the family Arenaviridae. Immunoglobulins were conjugated to the ribosome-inactivating protein, gelonin, through a disulfide linkage to form the immunotoxins. Immunotoxins were produced utilizing monoclonal antibodies, immunoglobulin-binding proteins and hyperimmune sera. An immunotoxin consisting of hyperimmune rabbit sera conjugated with gelonin displayed strong antiviral activity against Pichinde virus, as did a protein G-gelonin indirect immunotoxin in combination with nonconjugated hyperimmune sera. Hyperimmune rabbit sera conjugated with gelonin caused no detectable cytotoxicity in noninfected Vero cells as measured by [$^3$H]leucine incorporation. The 50% effective dose for the immunotoxin was 0.018 μM compared with 86 μM for ribavirin.

Immunotoxin; Gelonin; Pichinde virus; Antiviral; Arenavirus; Targeted delivery

## Introduction

Currently available antiviral drugs usually do not differentiate between infected and normal cells in their toxic effects. Many of these antiviral drugs are relatively ineffective and must be administered in such high doses that adverse side effects

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become a major concern. The effectiveness of such antiviral drugs would be enhanced by delivering them specifically to infected cells, thereby altering the distribution ratio compared with that achieved without targeting. Antibody-mediated targeted delivery is possible when the antigens expressed on the surface of infected and normal cells differ. Many viral infections are characterized by the expression of virus-specific antigens on the surface of infected cells, which may serve as target antigens. By using antibodies that bind to infected cells it may be possible to deliver drugs specifically to the infected cells and achieve a higher therapeutic index than is possible without the use of targeted delivery (Barnett, 1987). Since most antiviral drugs must be present in relatively high concentrations to exert an antiviral effect, multiple loading technologies and compatible delivery systems need to be developed before antibody-mediated delivery of antiviral drugs is likely to be effective. For this reason, we studied immunotoxins (ITs) as an antiviral therapy because, unlike the nucleoside antiviral drugs, anti-ribosomal toxins are highly active even at extremely low molar concentrations.

The approach to antiviral therapy described in this report utilizes the targeted delivery of the cytotoxin gelonin to interrupt protein synthesis in virus-infected cells. Gelonin, a 30 kDa, single-chain glycoprotein obtained from the seeds of *Gelonium multiflorium*, inactivates the 60 S ribosomal subunit in a catalytic manner, thus shutting down protein synthesis in eukaryotic cells (Stirpe et al., 1980). Theoretically, one molecule of gelonin inside of a cell would eventually stop all protein synthesis in that cell. Unlike other ribosome-inactivating toxins such as abrin and ricin, which consist of a toxic A chain and a B chain that binds to the cell surface and affords the mechanism by which the A chain enters the cell (Vitetta and Uhr, 1985), gelonin is composed solely of an A chain (Stirpe et al., 1980; Falasca et al., 1982). Because gelonin has no inherent mechanism for entering cells, it is much less toxic in cell culture and in vivo unless provided with a mechanism for entering cells. Immunotoxins enter cells by receptor-mediated endocytosis (Collier and Kaplan, 1984) and have been widely used on an experimental basis for killing cancer cells (Blythman et al., 1981; Bumol et al., 1983; Arnon et al., 1985; Vitetta and Uhr, 1985; Scott et al., 1987). Pincus et al. (1989) and Matsushita et al. (1990) have described ricin-immunotoxins which selectively kill HIV-infected cells. This report examines the antiviral activity of gelonin-ITs targeted against Pichinde virus (PCV)-infected cells.

Immunotoxins consisting of anti-PCV antibodies attached to gelonin through a disulfide linkage were prepared. The heterobifunctional, cleavable cross-linker *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was used to introduce dithiopyridyl groups into the immunoglobulin, whereas gelonin was modified with 2-iminothiolane. The thiolated gelonin was then allowed to react with the dithiopyridyl-substituted immunoglobulin in an exchange reaction to produce the disulfide bond of the IT (immunoglobulin–NH·CO·$CH_2$·$CH_2$·S–S·gelonin) (Cumber et al., 1985; Lambert et al., 1985).

## Materials and Methods

*Cells and media*

African green monkey kidney cells (Vero) obtained from the American Type Culture Collection (ATCC, Rockville, MD) were cultured in minimum essential medium (MEM; GIBCO Laboratories, Grand Island, NY) containing 5% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Cells growing as monolayers in cell-culture flasks were detached using trypsin and seeded into 24-well plates at $2 \times 10^5$ cells per well. For experiments utilizing 96-well plates, cells were seeded at $6 \times 10^4$ cells per well.

*Virus*

The An 4763 strain of Pichinde virus (obtained from Dr Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC) was used throughout these studies.

*Ribavirin and gelonin*

Ribavirin was provided by ICN Pharmaceuticals (Costa Mesa, CA). Gelonin was obtained from Pierce Chemical Co. (Rockford, IL).

*Antisera and monoclonal antibodies*

Hyperimmune antisera were obtained from rabbits immunized with purified PCV. The derivation of the hybridoma cell line that secretes the anti-PCV monoclonal antibody (mAb) designated PC4.9A6 and the production of ascites fluids were described previously (Burns et al., 1988). The PC4.9A6 monoclonal antibody utilized in these studies was of the $IgG_{2a}$ isotype. The immunoglobulins from ascites fluids and sera were purified by precipitation with ammonium sulphate and further purified by affinity chromatography on protein A linked to Sepharose (Bio-Rad Laboratories, Richmond, CA). The IgG concentration in the final product was determined by optical density assuming $E_{280}$ for 1 mg/ml (1 cm path) of 1.35. The antigen-binding activity of the antibodies and ITs was assayed by indirect immunofluorescence on PCV-infected Vero cell cultures. The presence of anti-PCV antibody was detected by immunostaining with fluorescein-labeled goat antibody specific for mouse or rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN).

*Preparation of immunotoxins*

The buffers and reagents used in the syntheses of the ITs were prepared as follows. SPDP (10 mM) in absolute ethanol and 2-iminothiolane (0.1 M) in 1.0 M triethanolamine-HCl buffer (1 M TEA buffer) were prepared just before use. The 1

M TEA buffer was prepared by mixing equal volumes of 1 M triethanolamine-HCl and 1 M triethanolamine free base. SPDP and 2-iminothiolane were obtained from Pierce Chemical Co. Recombinant protein G was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphate saline buffer (PSB) was: 0.1 M $Na_2HPO_4$, 0.1 M NaCl, 1 mM EDTA, pH 7.5. Phosphate-buffered saline (PBS) was: 0.01 M $Na_2HPO_4$, 0.15 M NaCl, pH 7.2. TEAE buffer was of the composition: 60 mM triethanolamine-HCl, 1 mM EDTA, pH 8.0. Bis-tris/acetate buffer was: 5 mM bis-tris, 50 mM NaCl, 1 mM EDTA, pH 5.8. The TEAE buffer and the bis-tris/acetate buffer were flushed by slowly bubbling argon through the buffers for 2 h; the argon flushed buffers were stored in tightly sealed bottles under argon. A stock solution of 20 mM iodoacetamide was prepared by dissolving iodoacetamide in doubly distilled water.

SPDP was used to introduce 2-pyridyl disulfide residues onto immunoglobulins (Cumber et al., 1985) and protein G. To 10 mg of purified immunoglobulin at a concentration of 5 mg/ml in PBS or to 5 mg of recombinant protein G in PBS at 2.5 mg/ml was added 225 μg of SPDP (as a 10 mM solution) with rapid mixing. Reaction mixtures were gently mixed at 37°C for 60 min. Excess SPDP and low-molecular-weight reaction products were removed from the reaction mixtures by gel chromatography on Sephadex G25 PD-10 columns (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated with PBS. The dithiopyridyl groups so introduced were stable for several days at pH 7.0 when stored at 4°C.

Sulfhydryl groups were introduced into gelonin utilizing 2-iminothiolane (Lambert et al., 1985). Just prior to the conjugation reaction, gelonin (5 mg) was dissolved in 2 ml of argon-flushed TEAE buffer. The 2-iminothiolane stock solution was then prepared. To the 2 ml of gelonin was added sufficient 0.1 M 2-iminothiolane to make the reaction mixture 2.5 mM in 2-iminothiolane. The reactants were gently mixed under an argon atmosphere for 90 min at 4°C. Excess 2-iminothiolane was removed from the thiolated gelonin by gel chromatography on Sephadex G25 PD-10 columns equilibrated and eluted with argon-flushed 5 mM bis-tris/acetate buffer, pH 5.8. The thiolated gelonin was used immediately for reaction with the 2-pyridyl disulfide-containing immunoglobulins to form the ITs.

To 10 mg (3.5 ml) of the 2-pyridyldithio-derivatized antibodies or 5 mg (also in 3.5 ml) of the derivatized protein G was added 1.15 ml of the thiolated gelonin (approximately 1.5 mg of gelonin) in 5 mM bis-tris/acetate buffer, pH 5.8. The pH of the reaction mixture was adjusted to pH 7.0 with TEAE buffer. The reaction mixture was then incubated at 4°C for 20 h. At the end of the conjugation reaction, the remaining free sulfhydryl groups were blocked by addition of 0.5 ml of 20 mM iodoacetamide followed by 1 h of incubation at 25°C. Nonconjugated gelonin was removed from the reaction mixture by dialysis against PBS using Spectropore 50000 MWCO membranes (Spectrum Medical Industries, Inc., Los Angeles, CA), followed by chromatography (Cumber et al., 1985) on Sephacryl S300 (Pharmacia LKB Biotechnology Inc.) (1.5 × 70-cm column) equilibrated with PBS. The protein concentrations were measured by optical density at 280 nm. The recovery of derivatized antibody and gelonin in the IT was 40% to 60%. Examination of the

ITs by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions revealed that over 85% (based on intensity of bands after Coomassie staining) of the protein in the IT fractions was present in the form of conjugates containing a single gelonin and immunoglobulin or protein G per IT.

*Cell-free protein synthesis*

A cell-free protein synthesis system was used to measure gelonin activity. Nuclease-treated rabbit reticulocyte lysate and L-[3,4,5-$^3$H]leucine from New England Nuclear, Inc. (Boston, MA) and untreated rabbit reticulocyte lysate from Promega, Inc. (Madison, WI) were used. The general details of the reaction conditions were as described by Pelham and Jackson (1976). Thin layer chromatography (TLC) on silica gel-impregnated glass-fiber sheets (Gelman Sciences Inc., Ann Arbor, MI) cut into 7 × 50-mm strips was used to separate the free and incorporated [$^3$H]leucine. The TLC solvent was prepared by mixing 15 ml of methanol, 5 ml of acetic acid and 30 ml of 15% (w/v) trichloroacetic acid (TCA). A few min before use, mRNA was prepared from 30 µl of untreated reticulocyte lysate to which was added 1 µl of 0.1 M EGTA to bind any Ca (II) that would reactivate the nuclease present in the nuclease-treated lysate. The resulting EGTA concentration was 6.25 mM. EGTA (0.1 M) was prepared by dissolving EGTA in sterile distilled water and adjusting to pH 7 with 1 N NaOH.

*Effect of reduced and nonreduced immunotoxins upon cell-free protein synthesis*

The assays were performed utilizing controls of translation reaction mixtures with and without mRNA, both without IT. The IT was reduced with 100 mM dithiothreitol by adding 10 µl of 1 M dithiothreitol to 0.1 ml of the IT and incubating for 30 min at room temperature. Reduced and nonreduced IT were then diluted in PBS containing bovine serum albumin fraction V (BSA) at 0.1 mg/ml. By comparing the extent of translation with varying dilutions of reduced and nonreduced IT in the translation reaction mixture to the extent of incorporation in controls, the effect of IT was determined. After incorporation periods of 15, 30 and 60 min, aliquots of translation reaction mixtures were quenched by mixing with 1 M Tris, pH 10.7. The high pH deacylated [$^3$H]leucyl-tRNA present in the mixture. The incorporated and free [$^3$H]leucine were separated by TLC. The incorporated amino acids stayed at the origin while the free moved with the front. Developed strips were cut in half (horizontally) to allow determination of both free and incorporated counts on each strip. Halves were placed in separate vials and [$^3$H]leucine was determined by liquid scintillation counting.

*In vitro antiviral assays based upon virus yield for evaluation of immunotoxins*

Vero cell cultures in 24-well plates were inoculated with PCV at a multiplicity of infection (moi) of 0.0001. At 20 h p.i., cells were rinsed once with cold (4°C) MEM, and 250 µl of cold MEM with varying concentrations of first antibody, such

as hyperimmune rabbit sera (HRS) towards PCV or direct IT, such as HRS-gelonin was added. Incubation was for 1 h at 4°C, followed by 3 h at 25°C, the cell sheets were rinsed twice with cold MEM, and 1 ml of MEM was added to each well. For the indirect immunotoxin experiments, dilutions of protein G-gelonin were then added (0.5 ml/well) for a second incubation of 3 h at 25°C before rinsing twice with MEM and adding MEM to all wells. Subsequent incubation was at 37°C. At 4 days p.i., the supernatant fluids were removed and saved for virus yield assays (duplicate assays on each of 3 cultures for each treatment condition). The antiviral activity of each conjugate was determined using reduction in the number of immunofluorescent foci (IF) as the scoring parameter in a manner analogous to measuring activity as a reduction in cytopathogenic effect. The antiviral activity was expressed as the concentration of drug required to reduce the IF score by 50% ($ED_{50}$). The assay utilized fluorescein-labeled anti-PCV mAbs for immunostaining of PCV infected Vero cells. The PCV assay and the preparation of the fluorescein-labeled antibodies was as described by Burns et al. (1988). Immunostained cells were detected by epifluorescence microscopy. The time-course experiments which utilized HRS-gelonin, employed parallel cultures which were treated at 20 h p.i. and then at each time point 3 cultures were harvested for virus yield assays commencing at 24 h p.i. (4 h post treatment) and each day thereafter for 5 days.

## Results

*Effect of immunotoxins upon protein synthesis in a reticulocyte lysate system*

To determine whether the gelonin activity had survived the conjugation procedure the effect of reduced and nonreduced PC4.9A6-gelonin upon protein synthesis in a reticulocyte lysate system was examined. Varying dilutions of the IT were added at the initiation of the translation reaction and samples were removed at 15, 30 and 60 min. The effect of reduced and nonreduced IT upon protein synthesis at 15 min is shown in Fig. 1. The data reflect [$^3$H]leucine incorporation as a percentage of that of the control without IT. Similar results were obtained for 30 min and 60 min incorporation periods. A 3 µl aliquot of the control (with mRNA but no IT) reaction mixture resulted in 150000 to 300000 cpm of incorporated [$^3$H]leucine depending on the length of the incorporation reaction. The maximum incorporation was 60% of the available [$^3$H]leucine.

The concentration of IT in the undiluted starting material was 330 µg/ml; the concentration with respect to gelonin was 53 µg/ml (1.8 µM). The concentration of reduced IT required to inhibit the incorporation of [$^3$H]leucine by 50% was 3.3–8.7 $\times 10^{-4}$ µg/ml, depending on the length of the incorporation reaction. In comparison, 200–1000-fold more of the nonreduced IT was required for the same degree of inhibition (Table 1). Greater than 75% of the original gelonin activity was displayed following reduction of the IT. Thus, the gelonin in the PC4.9A6-gelonin IT was present in a potentially active form. Similar results were obtained with the HRS-gelonin and protein G-gelonin immunotoxins described below (data not shown).

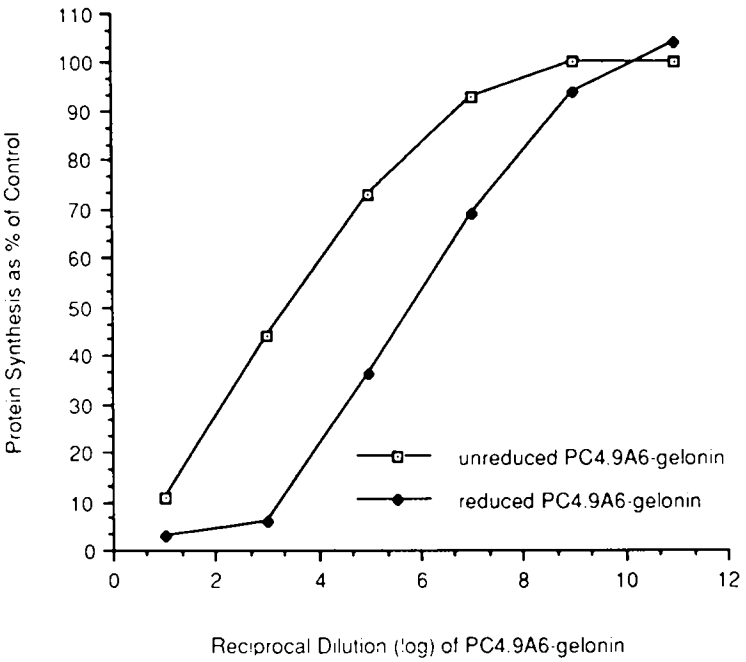


Fig. 1. Effect of native and reduced immunotoxin PC4.9A6-gelonin upon protein synthesis in a reticulocyte lysate cell-free translation system as described in Materials and Methods. Varying dilutions of the reduced or nonreduced immunotoxin were added at the initiation of the translation reaction. The reciprocal dilutions of immunotoxin are on a $\log_{10}$ scale. The data reflect [$^3$H]leucine incorporated after 15 min as a percentage of that of the control without immunotoxin. The concentration of gelonin in the undiluted immunotoxin was 1.8 μM.

*Effect of immunotoxins on Pichinde virus infection of Vero cells*

The effect of direct and indirect ITs on PCV yields in Vero cell cultures was evaluated. The direct immunotoxin (HRS-gelonin) was produced by conjugating gelonin to IgG isolated from the serum of rabbits hyperimmunized towards PCV, these purified IgGs, in the absence of complement, had no detectable neutralizing activity towards PCV. The indirect immunotoxin (IIT) consisted of gelonin attached to recombinant protein G, which binds to the Fc region of immunoglobulins.

PCV-infected cell cultures (moi of 0.0001, approximately 20 foci of infected cells per well) were treated with IT or antibody/IIT combinations at 20 h p.i. and the infections were allowed to proceed. At 4 days p.i., the culture fluids were removed for virus assays. In these experiments, the untreated control cultures yielded PCV titers of $2 \times 10^5$ fluorescent cell-forming units per ml; virus yields in terms of percent of control are summarized in Fig. 2. Only HRS in combination with IIT or HRS conjugated directly to gelonin produced antiviral effects: virus yield reductions of 96% and 80%, respectively. The other treatment combinations failed to reduce virus yields.

TABLE 1

Gelonin activity in disulfide-linked and reduced PC4.9A6-gelonin immunotoxin[a]

| Assayed following incorporation period (min) | 50% Inhibitory concentration[b] (μg/ml) | |
|---|---|---|
| | Disulfide-linked immunotoxin | Reduced[c] immunotoxin |
| 15 | $1.6 \times 10^{-1}$ | $8.7 \times 10^{-4}$ |
| 30 | $3.3 \times 10^{-1}$ | $3.3 \times 10^{-4}$ |
| 60 | $1.6 \times 10^{-1}$ | $8.7 \times 10^{-4}$ |

[a]Gelonin activity as measured by inhibition of [$^3$H]leucine incorporation in a cell-free protein synthesis system as described in Materials and Methods.
[b]50% inhibitory concentration was the concentration of immunotoxin that diminished the incorporation of [$^3$H]leucine by 50% after the indicated incorporation period.
[c]Reduction of the disulfide-linked PC4.9A6-gelonin with 100 μM dithiothreitol results in the release of free gelonin from the conjugate.

*Characterization and optimization of the antiviral activity of HRS-gelonin*

HRS-gelonin was chosen for subsequent evaluations and optimization. The characterization studies were designed to answer three questions: (1) the time course for the expression of antiviral activity, (2) the optimal time post-treatment for measurement of antiviral effect, and (3) the relationship between IT concentration, antiviral activity, and nonspecific cytotoxicity.

HRS-gelonin was added at 20 h p.i.; parallel cultures were harvested each day for 5 days. The greatest difference between virus yields from treated and nontreated cultures was at 48 h p.i. (Fig. 3). However, with the low moi used, there was considerable variation in virus yields, even from untreated cultures, at 48 h p.i. For that reason, 72 h p.i. was the optimum time for measuring virus yields. The effect of IT concentration upon the antiviral activity, measuring virus yields at 72 h p.i. is shown in Fig. 4. The $ED_{50}$ was 0.018 ± 0.005 μM (mean ± SD of 3 experiments). Toxicity experiments utilizing inhibition of protein synthesis in noninfected cells were run in parallel. There was no apparent toxicity over the concentration range evaluated. The antiviral activities of HRS-gelonin, ribavirin, and controls are summarized in Table 2.

## Discussion

For more than a decade, ITs have been used to kill cancer cells. Although the technologies and concepts for utilizing antibody-mediated delivery of toxins to virus-infected cells have been known and described for several years (Barnett, 1987), reports on the successful application of targeted toxins as antiviral agents are only very recent. Pincus et al. (1989) reported that antibody-ricin A chain conjugates inhibited protein synthesis and HIV production in HIV-infected cell cultures. A related approach that did not use antibody, was that of Till et al. (1988) who used rCD4-ricin A chain to kill HIV-infected cells. Another, very innovative approach utilized a *Pseudomonas* exotoxin-CD4 recombinant protein to mediate in

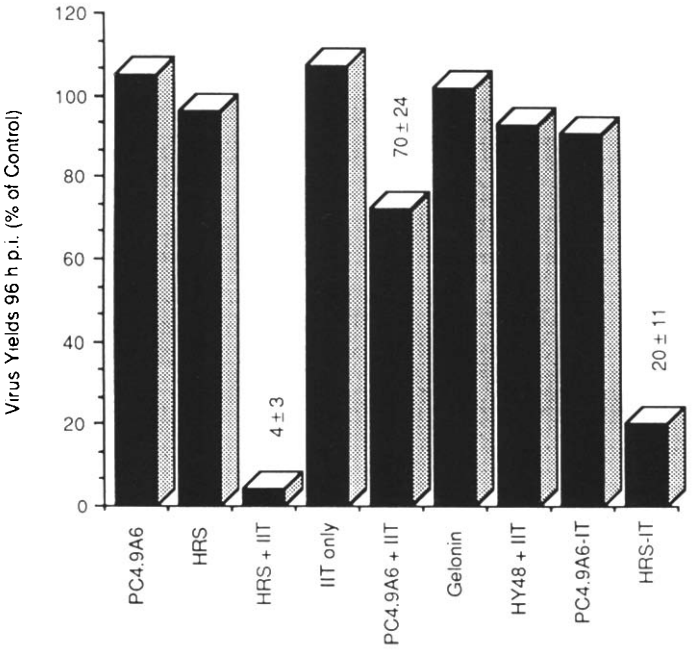


Fig. 2. Effect of immunotoxins on PCV yields. Vero cell cultures in 24-well plates were inoculated with PCV at an moi of 0.0001. Cultures were treated at 20 h p.i. with: PC4.9A6 (a non-neutralizing mAb that reacts with PCV antigens expressed on the surface of infected cells) at 5 μg/ml, HRS (IgG isolated from the serum of rabbits hyperimmunized with PCV) at 5 μg/ml, HRS+IIT (HRS followed by an indirect immunotoxin consisting of gelonin linked to protein G) at 5 μg/ml each, IIT only at 5 μg/ml, PC4.9A6 + IIT (PC4.9A6 followed by IIT) at 5 μg/ml each, gelonin (the toxin used to produce the immunotoxins) at 5 μg/ml, HY48 + IIT (HY48 is an isotype matched mAb that has no affinity for PCV or Vero cells) at 5 μg/ml each, PC4.9A6-IT (an immunotoxin consisting of PC4.9A6 conjugated to gelonin through a disulfide linkage) at 10 μg/ml, and HRS-IT (HRS conjugated to gelonin through a disulfide linkage also termed HRS-gelonin) at 6.4 μg/ml. Cultures were harvested at 96 h p.i. and assayed for PCV by immunofluorescent cell forming assay as described in Materials and Methods. The nontreated virus-infected control cultures yielded PCV titers of $2 \times 10^5$ fluorescent cell forming units per ml. Data are presented as virus yields in terms of percent of the nontreated virus-infected control cultures and represent the average of duplicate assays from each of 3 cultures for each set of conditions.

vitro killing of HIV-infected cells (Chaudhary et al., 1988). Matsushita et al. (1990) demonstrated the selective killing of HIV-infected cells in vitro with immunotoxins consisting of either ricin A chain or *Pseudomonas* exotoxin conjugated with a murine monoclonal antibody to the external envelope glycoprotein gp120.

This is the first report describing the antiviral activity of either antibody-gelonin or protein G-gelonin conjugates. Gelonin has been used in the construction of other types of ITs. Thorpe et al. (1981) produced disulfide linked gelonin-anti-Thy 1.1 mAb conjugates which were cytotoxic in vitro and in vivo to lymphocytes bearing the Thy 1.1 set of surface antigens. Stirpe et al. (1980) reported that 100 μg/ml of gelonin only slightly inhibited protein synthesis in intact HeLa cells, but when attached to concanavalin A, gelonin inhibited HeLa cell protein synthesis by 50%

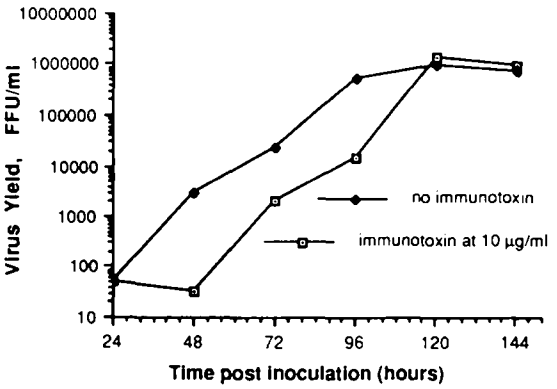


Fig. 3. Time course for the effect of immunotoxin (HRS-gelonin) upon Pichinde virus yields. Immunotoxin (10 µg/ml, equivalent to 0.056 µM) was added at 20 h p.i. to Pichinde virus-infected Vero cells in 6 different 24-well plates. Four hours post treatment (24 h p.i.) cells and fluids were harvested from first plate by freezing and thawing, one of the remaining plate cultures was harvested each day for 5 days and Pichinde virus titers determined by immunofluorescent cell forming assay and expressed as focus forming units/ml (FFU/ml). The initial moi was 0.0001 (20 focus forming units/well). Each data point is the average of assays of the culture fluids from each of six wells each of which was assayed in duplicate.

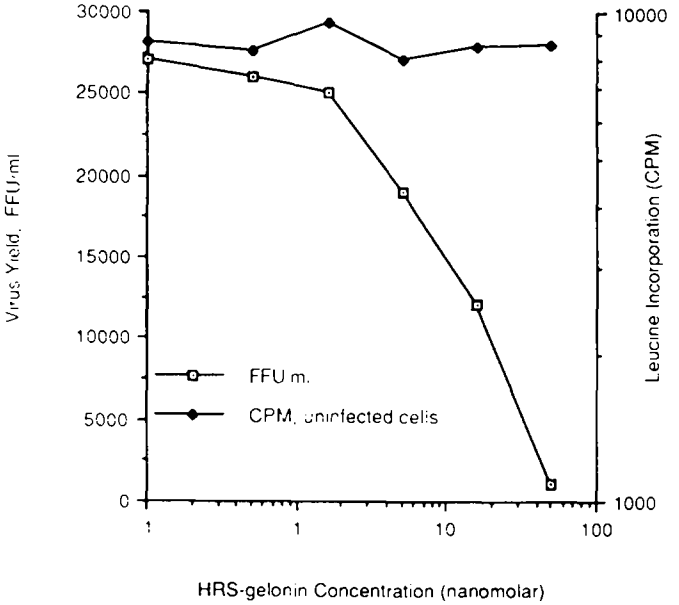


Fig. 4. Effect of immunotoxin (HRS-gelonin) concentration upon PCV yields from infected cells and upon protein synthesis in noninfected Vero cells. Vero cell cultures in 24-well plates were inoculated with PCV at an moi of 1 foci-forming unit (FFU) per 10000 cells, which produced 20 foci of infected cells per well by 20 h p.i. Immunotoxin was added at 20 h p.i. and Pichinde virus yields were determined at 72 h p.i.; virus yields are the average of 3 experiments. Toxicity experiment utilizing inhibition of [$^3$H]leucine incorporation (measured 48 h post treatment) in noninfected Vero cells was run in parallel.

TABLE 2

Comparison of antiviral activity of immunotoxins and controls against Pichinde virus in Vero cells[a]

| | $ED_{50}$[b] μg/ml, [μM] | $CD_{50}$[c] μg/ml, [μM] | Therapeutic index ($CD_{50}/ED_{50}$) |
|---|---|---|---|
| Ribavirin | 21 ± 4 [86] | 320 [1311] | 15 |
| HRS[d] | >350 | >350 | – |
| HRS-gelonin[e] | 3.2 ± 0.9 [0.018] | >160 [>0.9] | >50 |
| HY48-gelonin[f] | >160 | >160 | – |

[a]One treatment by adding test material to medium at 20 h p.i.
[b]Antiviral activity determined by measuring reduction in virus yield at 72 h p.i., $ED_{50}$ ± SD (average of 3 experiments).
[c]Toxicity determined by measuring reduction in [$^3$H]leucine incorporation in uninfected Vero cells at 48 h after treatment.
[d]HRS, IgG isolated from the serum of rabbits that had been hyperimmunized with Pichinde virus.
[e]HRS-gelonin, an immunotoxin consisting of HRS conjugated to gelonin through a disulfide linkage.
[f]HY48-gelonin, a negative control immunotoxin consisting of monoclonal antibody with no affinity for Pichinde virus or Vero cells conjugated to gelonin through a disulfide linkage.

at a gelonin concentration of 0.2 μg/ml. Lambert et al. (1985) examined the cytotoxicity of gelonin conjugates directed at human lymphocytes by measuring the [$^3$H]thymidine incorporation as an index of cytotoxicity. They found that the $ID_{50}$ for one of their gelonin conjugates on the appropriate target cells was 30 pM; a 30 picomolar solution of gelonin would contain gelonin at $1 \times 10^{-3}$ μg/ml.

Our initial studies utilized an IT produced by conjugating the mAb PC4.9A6 to gelonin. PC4.9A6 was selected because it bound to the surface of PCV-infected cells, but possessed no neutralizing or protective activity in vitro or in vivo. This latter quality is desirable, in that any protective effect associated with PC4.9A6-gelonin would be attributable to an IT effect and not an inherent protective activity associated with the antibody.

PC4.9A6-gelonin was examined to determine if the gelonin activity survived the conjugation procedure. Expression of maximal anti-ribosomal activity of the gelonin in PC4.9A6-gelonin occurred only after cleavage of the disulfide linkage, a reaction likely to occur in the reducing environment of the lysosomal vesicles inside a target cell (Trouet et al., 1982). Using a similar cell-free protein synthesis system, Lambert et al. (1985) reported that gelonin at $7.4 \times 10^{-4}$ μg/ml shut down protein synthesis within 15 min. In the experiments described in this report, the concentration of gelonin in the reduced IT required to inhibit incorporation of [$^3$H]leucine by 50% was $0.5–1.4 \times 10^{-4}$ μg/ml. Thus, the PC4.9A6-gelonin retained the potential toxicity of the gelonin.

Although PC4.9A6-gelonin possessed the ability to block protein synthesis in a cell-free system, the antiviral assays indicated no antiviral activity. Yet, it was shown that PC4.9A6-gelonin retained specific binding activity to PCV-infected cells. The possibility that monensin might enhance the activity of PC4.9A6-gelonin was examined. Casellas et al. (1985) reported that the in vitro activity of ITs composed of the purified A chain of ricin covalently attached to a mAb directed at an antigen present on mouse leukemia cells was greatly enhanced (7- to 10-fold) by

the carboxylic ionophore monensin. Treatment of cells with monensin disturbed the acidification of the lysosomes and greatly increased both the kinetics of cell-killing and the activity of the ITs. In our studies, there was no specific cytotoxicity associated with PC4.9A6-gelonin either without monensin or with monensin. The 50% inhibitory concentration for PC4.9A6-gelonin was 100 μg/ml under all conditions evaluated. In an effort to determine why the PC4.9A6-gelonin lacked antiviral activity, fluorescein-labeled PC4.9A6 was used to examine the binding and internalization of PC4.9A6 in PCV-infected cell cultures. Fluorescein-labeled PC4.9A6 was added to viable cultures of PCV-infected Vero cells which were then incubated at either 37 or 4°C and then examined using fluorescence microscopy. At both 37 and 4°C PC4.9A6 bound to the PCV-infected, but there was no evidence for internalization at either temperature. Failure to internalize could account for the failure of PC4.9A6-gelonin to display antiviral activity.

Additional antibodies, which might bind to viral antigens that were more readily internalized, were used in subsequent experiments. Experiments using an indirect immunotoxin, protein G-gelonin, and a direct IT, HRS-gelonin, were conducted. It was thought that the indirect approach might facilitate identification of antibodies suitable for targeted delivery. The use of polyclonal antisera was intended to provide binding to a large number of PCV antigens on the infected cells and thus increase the chances for binding to a readily internalized antigen. When anti-PCV polyclonal antisera were used for both direct and indirect IT approaches, the virus yield data indicated a strong antiviral effect. The success with the indirect immunotoxin approach suggests that protein G-gelonin indirect immunotoxins could be used to screen for mAbs suitable as targeted delivery vehicles.

The conjugates employing polyclonal antibodies for targeted delivery had very strong antiviral effects against PCV. One treatment with HRS-gelonin at 20 h p.i. reduced the virus yield by over 1000-fold (Fig. 4) with no detectable nonspecific cytotoxicity. The IgG from the HRS displayed little or no PCV-neutralizing activity ($SN_{50}$ of less than 1:10) in the absence of complement (Table 2 and Fig. 2). Thus, the antiviral activity was associated with the toxin portion of the HRS-gelonin and could not be attributed to neutralization by the HRS. The $ED_{50}$ for HRS-gelonin against PCV infection in Vero cells was 0.018 ± 0.005 μM. Pincus et al. (1989) reported an in vitro $ED_{50}$ of 0.004 μM for an anti-HIV-ricin A chain conjugate which was constructed with a monoclonal antibody. The HRS used to construct HRS-gelonin was not monospecific for PCV antigens; removal of the irrelevant immunoglobin molecules from HRS would lower the $ED_{50}$.

Ribavirin, the drug of choice for treating arenavirus infections (Stephen et al., 1980; Huggins et al., 1984), was the positive control in these studies. Ribavirin has an $ED_{50}$ of 6 μg/ml against PCV in Vero cells when treatment is at 30 min p.i. (Burns et al., 1988), but since surface-expressed PCV antigens are not expressed this early in an infection, the antiviral activities of HRS-gelonin and ribavirin were compared based on treatment at 20 h p.i. (Table 2). The $ED_{50}$ for HRS-gelonin was 4800-fold lower than that for ribavirin (0.018 μM compared with 86 μM). One of the major advantages envisioned for antibody-mediated delivery of antiviral substances is a reduction in nonspecific cytotoxicity (side-effects); the data in Table 2

indicate that this potential advantage may be realized. No cytotoxicity was found with HRS-gelonin concentrations of up to 0.8 μM. In the future, ITs produced with suitable mAbs may be even more specific than the HRS-gelonin in these studies. Although additional characterization and optimization studies remain to be completed, the anti-PCV ITs described in this report appear promising for use in developing antibody-mediated delivery of antiviral substances.

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