Published in final edited form as:
Antiviral Res. 2011 June ; 90(3): 143–150. doi:10.1016/j.antiviral.2011.03.175.

An Immunotoxin targeting the gH glycoprotein of KSHV for selective killing of cells in the lytic phase of infection

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Abstract

Amongst the pathologies associated with infection by Kaposi's sarcoma-associated herpesvirus (KSHV), multicentric Castleman's disease is distinctive for involvement of the lytic phase of the virus replication cycle. This B cell lymphoproliferative disorder has shown clinical responsiveness not only to generalized immunotherapy and cytotoxic chemotherapy, but also to inhibitors of herpesvirus DNA replication, consistent with the involvement of lytic phase of replication. These findings suggest that selective killing of virus-producing cells might represent a novel therapeutic strategy. We designed an immunotoxin, YC15-PE38, containing a single chain variable region fragment of a monoclonal antibody against KSHV glycoprotein H (gH) linked to the effector domains of *Pseudomonas* aeruginosa exotoxin A. Purified YC15-PE38 displayed highly selective and potent killing of a gH-expressing transfectant cell line (subnanomolar IC_{50}). The immunotoxin also strongly inhibited production of infectious KSHV virions from an induced chronically infected cell line, by virtue of selective killing of the virus-producing cells. Combination treatment studies indicated complementary activities between YC15-PE38 and the herpesviral DNA replication inhibitor ganciclovir. These results provide support for the development of anti-KSHV strategies based on targeted killing of infected cells expressing lytic phase genes.

Keywords

Kaposi's sarcoma-associated herpesvirus; human herpesvirus-8; immunotoxin; glycoprotein H; multicentric Castleman's disease; lytic infection; ganciclovir

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus 8) is a gammaherpesvirus initially discovered by DNA analysis of Kaposi's sarcoma (KS) biopsy tissues from AIDS patients (Chang et al., 1994). Beyond its initial etiological implication in KS, the virus was subsequently linked to two B-cell lymphoproliferative disorders (Cesarman and Knowles, 1997; Chang and Moore, 1996): primary effusion lymphoma (PEL) (Cesarman et al., 1995) and multicentric Castleman's disease (MCD) (Soulier et al., 1995). Typical of the *Herpesviridae* (Pellett and Roizman, 2007), KSHV infected cells can exist in both latent and lytic phases of the infection cycle, with marked variations observed in different KSHV-associated pathologies. Thus in KS and PEL, the virus persists

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extensively in the latent phase, whereas in MCD, lytic replication is prominent (Burbelo et al., 2010; Marcelin et al., 2007). The likely differences in gene regulation patterns and associated cellular signaling pathways for these distinct pathologies suggest that effective treatment strategies might differ (Casper, 2008; Schulz, 2006; Sullivan et al., 2008).

Castleman's disease is a relatively rare lymphoproliferative syndrome first described over a half century ago (Castleman and Towne, 1954). It can occur in either the relatively benign unicentric form or the more aggressive MCD characterized by diffuse peripheral lymphadenopathy and systemic symptoms (Bower, 2010; Oksenhendler, 2009; Stebbing et al., 2008). Like the other hallmark KSHV-associated diseases, MCD is commonly observed in the context of HIV co-infection. The syndrome involves episodic remission and relapse, with active disease associated with abnormally large plasmablasts in lymph nodes as well as high levels of KSHV DNA in blood. Curiously, while antiretroviral therapy has been associated with a marked reduction of incidence of AIDS-KS as well as regression of KS lesions (Dittmer et al., 2005), recent reports have indicated an increasing incidence of HIV-associated MCD despite highly active antiretroviral therapy (HAART) and the associated improvements in CD4 cell counts (Kenyon et al., 2007; Mylona et al., 2008; Powles et al., 2009). Prognosis and median survival time remain poor, with frequent progression to non-Hodgkins lymphoma (Mylona et al., 2008; Stebbing et al., 2008).

At present there are no standardized treatment regimens for MCD. Beneficial clinical effects have been reported with several classes of agents, both nonherpesvirus-specific and herpes-directed (Bower, 2010; Oksenhendler, 2009; Stebbing et al., 2008). The former include anticancer treatments such as steroids and cytotoxic chemotherapy (single agent or combination) (Herrada et al., 1998), as well as immunotherapy with monoclonal antibodies (mAbs) such as rituximab against the B-cell antigen CD20 as reported in cohort studies (Bower et al., 2007; Gerard et al., 2007) and Tociliizumab against the IL6 receptor (Matsuyama et al., 2007). Aggressive combination treatment with chemotherapy and rituximab has shown efficacy in recent case reports of advanced MCD (Bestawros et al., 2008; Schmidt et al., 2008). Herpes-directed treatments have been based on the *in vitro* activities of several inhibitors of herpesvirus DNA polymerase against KSHV (Oksenhendler, 2009; Stebbing et al., 2008). Promising findings reported in humans include a case study indicating that ganciclovir promoted symptomatic relief and reduction of KSHV DNA load in plasma of MCD subjects (Casper et al., 2004) followed by a randomized double-blind placebo controlled crossover trial demonstrating that oral valganciclovir inhibited KSHV replication in the oral mucosa of asymptomatic subjects as judged by reduced frequency and quantity of KSHV shedding (Casper et al., 2008). However the dose-limiting toxicities of these drugs (Andrei et al., 2008) may compromise their long-term use for management of MCD.

We have been pursuing antiviral strategies directed at targeted killing of infected cells based on their surface expression of virus-encoded gene products. This concept is analogous to approaches under active development in the cancer field, using antibodies or ligands to target cytotoxic payloads to selectively kill cells expressing tumor-associated antigens (Sharkey and Goldenberg, 2008). In particular, several groups including ours initially developed recombinant protein immunotoxins generated from bacterial and plant protein toxins that selectively kill HIV productively infected cells based on their surface expression of the HIV Env glycoprotein (Aullo et al., 1992; Chaudhary et al., 1988; Pincus et al., 1989; Till et al., 1988). We have argued that improved versions of such agents might provide a critical complement to suppressive antiretroviral therapy in efforts to deplete persisting infected cell reservoirs (Berger and Pastan, 2010). In the present report, we describe an immunotoxin directed against glycoprotein H (gH) of KSHV, for possible use in treatment of pathologies such as MCD in which cells in the lytic phase of the viral replication cycle

play a significant role. Combination treatment of KSHV-infected cells with the immunotoxin plus a herpesvirus replication inhibitor is also examined.

2. Materials and Methods

2.1 Cells

293 cells (derived from human embryonic kidney, Freestyle 293F subclone from Invitrogen) were cultured in DMEM with 10% fetal bovine serum (FBS), 2 mM L-glutamine and amino acid supplement. Vero cells (derived African green monkey kidney) harboring recombinant KSHV rKSHV.219 (Vieira and O'Hearn, 2004) (herein referred to as Vero-219, generously donated by Jeffrey Vieira, University of Washington) were cultured in DMEM supplemented with 10% (FBS), 2mM L-glutamine and 5ng/ml puromicin.

2.2 Immunotoxin plasmid construction

Immunotoxins composed of single chain variable fragments (scFvs) of mAbs linked to the translocation and ADP-ribosylation domains of *Pseudomonas aeruginosa* exotoxin A were designed by standard methods (Pastan et al., 2003). Total cellular RNAs from 10_7 YC15 hybridoma cells were isolated by the Qiagen RNA isolation kit as described in the product manual. 2.5 μg of total RNA and isotype-specific V_H hinge primer (MG2a-Hinge: 5′-TCT GGG CTC AAT TTT CTT GTC CAC C-3′) or V_L hinge primer (MK-Edge: 5′-CTC ATT CTT GTT GAA GCT CTT GAC AAT-3′) were used to set up the reaction for cDNA synthesis as described in the SMART RACE cDNA amplification kit (Clonetech, Palo Alto, CA). The prepared cDNAs were used as the template to set up the 5′RACE PCR with 10 pmol of isotype specific primers (MG2a-PCR: 5′-AGG GGC CAG TGG ATA GAC CGA TGG GGC TGT-3′, MK-PCR: 5′-GGA TGG TGG GAA GAT GGA TAC AGT TGG TGC AGC-3′). The PCR products were cloned into pCR2.1-TOPO vector through TOPO TA cloning kit (Invitrogen). At least 10 clones for each chain were sequenced. The sequences were analyzed and aligned according to the Ig BLAST program using the Kabat database (http://www.ncbi.nlm.nih.gov/igblast/).

The primers used to synthesize V_H and V_L fragments were designed according to the nucleotide sequences alignment. The primer pair used to amplify the heavy chain Fv region were 5′ H15-NdeI (5′-AAA CAT ATG GAG GTT CAG CTC CAG CAG TCT-3′) and 3′ H15-Linker (5′-TCC AGA TCC GCC ACC ACC TGA TCC GCC TCC GCC TGA GGA GAC GGT GAC TGA GGT-3′). The primer pair used to amplify the light chain Fv region were 5′L15-Linker (5′-TCA GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAC ATC CAA ATG ACA CAA TCT -3′) and 3′ L15-HindIII (5′-GGA AGC TTT CCG TTT GAT TTC CAG CCT GGT -3′). The PCR products encoding the V_H and V_L domains of mAb YC15 connected by a 15 amino acid linker $(Gly_4Ser)_3$ were generated by fusion PCR using the purified individual V_H and V_L PCR fragments through the primers 5′ H15-NdeI and 3′ L15-HindIII. The PCR product was then digested with *Nde* I and *Hind* III and the resulting fragment was used to replace the *Nde* I - *Hind* III fragment from expression plasmid pTK21.8 encoding 3B3(Fv)-PE38 (Bera et al., 1998). The resulting YC15-PE38 expression plasmid is designated pYC51. We also produced an expression plasmid designated pYC52 encoding a negative control protein YC15- $PE38^{E553D}$, a variant of YC15-PE38 containing the E553D mutation in the PE38 domain that inactivates its ADP ribosylation activity (Douglas and Collier, 1987). A control immunotoxin, BL22-PE38 directed against the CD22 antigen (Kreitman et al., 2001), was generously provided by Ira Pastan (NCI, NIH).

2.3 Immunotoxin expression and purification

Immunotoxin proteins were expressed and purified as described previously (Pastan et al., 2003). Briefly, the immunotoxin expression plasmids were transformed into *E. Coli* BL21 (DE3). The bacterial cultures were induced with isopropyl-1-thio-β-d-galactopyranoside (IPTG, 1 mM final concentration) for 2 hr to express the recombinant proteins. The insoluble inclusion bodies were isolated by centrifugation steps, solubilized in 6M guanidine HCl and subsequently reduced by dithioerythritol. The solubilized reduced proteins were refolded in the refolding solution containing oxidized glutathione and L-arginine. After the refolding step the protein was dialyzed to remove guanidine hydrochloride and purified by anion exchange chromatography using Q-Sepharose and Mono-Q (Amersham Pharmacia Biotech), and by size exclusion chromatography (TSK3000; TOSOH, Tokyo, Japan). The proteins were analyzed by SDS-PAGE under reducing conditions. Protein concentrations of the purified immunotoxins were determined by a protein assay (Bio-Rad) with BSA as the standard.

2.4 Establishment of KSHV gH/gL stable transfectant cell line

Full length KSHV gH and gL sequence were PCR amplified from plasmids pJK2-gH and pJK3-gL (Kaleeba and Berger, 2006) with the following primers: 5′gH-*BamH*I (5′-ATT GGA TCC ACC ATG CAG GGT CTA GCC TTC TTG-3′), 3′gH-*Hind*III (5′-AAT AAG CTT CTA ATA AAG GAT GGA AAA CAG-3′), 5′gL-*Not*I (5′-AAT GCG GCC GCC ATG GGG ATC TTT GCG CTA TTT-3′) and 3′gL-*Xho*I (5′-AAT CTC GAG TTA TTT TCC CTT TTG ACC TGT GTG-3′). The gL fragment was cloned into the *Not* I and *Xho* I sites of pBudCE4.1 vector (Invitrogen) under the EF-1α promoter. The gH fragment was subsequently cloned into the *Hind* III and *Bam*H I sites of the same construct under CMV promoter. The resulting gH/gL expression plasmid is designated pYC50. 293 cells were transfected with pYC50 and selected by Zeocine antibiotics for at least two weeks. Cell lines stably expressing KSHV gH/gL were established by cell cloning; the cloned 293-gH/gL transfectant cell line used in this report is designated YC-293-gH/gL-clone 5.

2.5 Flow cytometry analysis

YC15-PE38 or 3B3(Fv)-PE38 immunotoxins were labeled with Alexa fluor 488 according to the manufacturer instruction (Alexa Fluor 488 microscale protein labeling kit, Invitrogen). 5×10^5 cells per sample were washed with PBS and blocked with 3%BSA in PBS at 4°C for 30 mins. The cells were incubated with different concentrations of labeled immunotoxins at 4°C for 1h. After washing, each sample was fixed with a 2% paraformaldehyde and flow cytometry analysis was performed with a FACS Scan (Becton Dickinson Co.).

2.6 Direct Cytotoxicity assay

WST-8 assay cell counting kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) was used to measure the cytotoxicity activities of the immunotoxins against the designated cells. Briefly, 5×10^3 cells/well were incubated with various concentrations of immunotoxins in a 96-well plate for 3 days. CCK-8 reagent (10 μl) was added to each well and the plate was incubated at 37°C incubator for 2 hours. Absorbance at 450nm was read. Cycloheximide (10 μg/ml) was used as a control for complete killing. All values were normalized to untreated controls.

2.7 Inhibition of infectious virus production assay

Vero-219 cells were seeded at 2×10^5 cells/well in a 24-well plate format. The cells were infected with recombinant baculovirus BacK50 (Vieira and O'Hearn, 2004) (from Jeffrey Vieira) at M.O.I. 100 for 3 hrs and washed one time with PBS. Then complete medium supplemented with the 1.25 mM Sodium Butyrate and different concentration of

immunotoxin was added into the cells. 24hrs later, the medium containing sodium butyrate was removed and replaced with complete medium containing the same immunotoxin concentrations. At 3 days post induction, the supernatants were harvested and assayed for infectious KSHV virions. Aliquots (0.2 ml) of supernatants were spin-inoculated (1500 RPM, 30 min) onto fresh 293 cells in 24 well plates (2×10^5 cells/well, 0.5 ml total volume); after incubation at 37°C for 3 hours, the inoculum was replaced with 0.5 ml fresh medium and the plates were incubated at 37°C for 2 days. The number of infected target cells was then determined by flow cytometry; infectivity is expressed as percentage of target cells that scored GFP-positive.

3. Results

3.1 Design and production of KSHV gH-targeted immunotoxin

We chose a recently developed murine hybridoma designated YC15 for immunotoxin production, since our analyses indicated that the corresponding mAb binds to gH expressed on the surface of gH transfectant cell lines and neutralizes infection by KSHV virions (Y. Cai and E. A. Berger, unpublished). From cDNA clones of the variable light and heavy chain regions, we designed an scFv and linked the sequences to those encoding PE38, a *Pseudomonas aeruginosa* exotoxin A construct lacking the native N-terminal cell binding domain but containing the domains involved in internalization and cell killing by inhibition of protein synthesis via ADP-ribosylation of elongation factor 2. The single chain recombinant immunotoxin, designated YC15-PE38, is shown schematically in Fig. 1A. The protein was expressed in *E. coli* and purified from inclusion bodies (see Materials and Methods). SDS-PAGE coupled with Coomassie Blue staining (Fig. 1B) indicated that YC15-PE38 was readily detectable in the total cell extract (compare lanes 2 and 3) and was the major component in the inclusion body preparation (lane 4). The purity of the final product (lane 5) was ≥ 95%. We also expressed and purified the negative control protein YC15-PE38^{E553D}, which lacks cytotoxic activity due to an inactivating mutation in the PE38 moiety.

3.2 Binding of YC15-PE38 to cell surface gH

We used flow cytometry to test the ability of the immunotoxin to bind to gH expressed on the cell surface (Fig. 2). We observed dose-dependent binding of YC15-PE38 to a KSHV gH/gL stable transfectant of 293 (panel A). Specificity was demonstrated by the lack of binding of this immunotoxin to parental 293 cells lacking gH (panel B), and of an irrelevant immunotoxin [BL22-PE38, which targets CD22] to either cell type (panels C and D).

3.3 Selective killing of KSHV gH-expressing cell lines by YC15-PE38

We tested the cytotoxic activity of YC15-PE38 against various cell lines. As shown in Fig. 3, the immunotoxin promoted dose-dependent killing of a 293 transfectant cell line expressing KSHV gH/gL. The cytotoxicity was quite potent, with an IC^{50} of 10 ng/ml (corresponding to 150 pM); this compares favorably with reported potencies of PE-based immunotoxins directed at antigens over-expressed on tumor cells (Wolf and Elsasser-Beile, 2009). Specificity was demonstrated in two independent control assays involving immunotoxin treatment over the same concentration range: YC15-PE38 showed negligible cytotoxicity for parental 293 cells (gH-negative), and an irrelevant PEbased immunotoxin (BL22-PE38 targeting CD22, which is not expressed on 293 cells) showed negligible cytotoxicity for both the parental 293 cells and the gH/gL-expressing 293 transfectant. The observed killing of the gH/gL transfectant cell line by YC15-PE38 was dependent on the cytotoxic activity of the PE38 moiety, as demonstrated by the lack of effect of YC15-PE38^{E553D}, which contains a point mutation that abolishes the ADP-ribosylation activity of the PE moiety.

3.4 Selective inhibition of infectious virus production by YC15-PE38

A prerequisite for the potential utility of YC15-PE38 is that it inhibit production of infectious virions from a producer cell; such an effect would reflect the ability of the immunotoxin to kill the infected cell before it produces the amount of virus that it would normally releases before succumbing to any cytopathic effect associated with the lytic phase of infection. In the experiments shown in Fig. 4, we examined the effects of immunotoxins on release of infectious virions from the Vero-219 cell line, which carries an episomal recombinant KSHV genome encoding infectious virions containing the eGFP gene linked to a strong constitutive cellular promoter. Virion release was stimulated by treatment for 3 days with sodium butyrate plus BacK50, a recombinant baculovirus encoding KSHV ORF 50 (RTA transcription factor) (Vieira and O'Hearn, 2004); the amounts of released infectious virus were quantitated by incubating the culture supernatants with susceptible 293 target cells and scoring the number of eGFP-positive cells after 2 days. Fig. 4 A shows that YC15-PE38 produced dose-dependent inhibition of infectious virus release, with potency much greater than that of the control BL22-PE38 immunotoxin. This inhibition was dependent on the cytotoxic activity of YC15-PE38 as demonstrated by the ineffectiveness of YC15-PE38^{E553D}, thus proving that the observed inhibition was due to specific killing of the producer cells rather than to direct neutralization by the YC15 scFv moiety. This latter possibility was unlikely based on other studies (Y. Cai and E. A. Berger, unpublished) with the YC15 mAb, which demonstrated that neutralization required much higher concentrations of the divalent antibody than those used in this experiment with the monovalent immunotoxin. The experiment shown in Fig. 4B demonstrates that the specificity of inhibition of infectious KSHV release by YC15-PE38 was observed at all time points over a week-long period of induction of the Vero-219 cells.

3.5 Effects of combinations treatment with YC15-PE38 and ganciclovir

As noted in the Introduction, the herpesviral DNA polymerase inhibitors ganciclovir and its prodrug valganciclovir have been shown to block KSHV replication *in vitro* and *in vivo*. These reports led us consider possible effects of combining such inhibitors with YC15-PE38. On the one hand, the two agents might show complementary anti-KSHV activity, since the former acts by blocking viral replication and the latter acts by killing cells that are already infected. On the other hand, since KSHV lytic gene expression is dependent on viral DNA synthesis (Lu et al., 2004), it is possible that a DNA polymerase inhibitor might compromise the activity of YC15-PE38 by blocking synthesis of gH, the target protein for the immunotoxin. We therefore compared the effects of each agent alone, and in combination over a range of concentrations of each agent, using inhibition of infectious virus production from induced Vero-219 cells as the assay. Fig. 5A shows dose-response curves of YC15-PE38 inhibition, alone and in combination with increasing concentrations of ganciclovir. The immunotoxin displayed activity over the entire range of ganciclovir; importantly as shown in the inset, the potency the immunotoxin was at least as great throughout the range of ganciclovir concentrations as it was when tested alone, as judged by the absence of increase in its IC_{50}. Fig. 5B shows the reciprocal experiment, i.e. dose-response curves of ganciclovir in the presence of increasing concentrations of YC15-PE38. A comparable effect was observed, i.e. ganciclovir was at least as potent in the presence of increasing doses of YC15-PE38 as it was alone, based on IC_{50}. Thus the results indicate that the two anti- KSHV agents show complementary rather than antagonistic activities.

4. Discussion

Amongst the pathologies associated with KSHV infection, MCD stands out virologically for its association with the lytic phase of the KSHV replication cycle, and clinically for its poor prognosis and increasing incidence in the HIV-infected population despite HAART. The

frontline therapeutic regimens for MCD, antineoplastic chemotherapy and rituximab, have shown some efficacy, but are not curative and are complicated by significant toxicities due to their generalized modes of action. Curiously rituximab was associated with not only remission of MCD symptoms, but also with flare-up of KS lesions in HIV-positive patients with prior KS (Bower et al., 2007; Gerard et al., 2007) and very recently with initial presentation of aggressive KS in an HIV-negative MCD patient (Law et al., 2010). Based on analysis of KSHV viral loads, it has been concluded that MCD relapse after a positive rituximab response was due not to the outgrowth of resistant variants, but instead to the failure to completely eliminate virus from B cells, thereby allowing resumption of lytic infection (Powles et al., 2007). These results, coupled with the promising clinical findings with herpesvirus DNA polymerase inhibitors described in the Introduction, highlight the need for therapeutic agents that act directly and specifically against KSHV, particularly in the lytic phase.

The results presented herein demonstrate that the YC15-PE38 immunotoxin displays highly potent and selective cytotoxicity against cells expressing surface KSHV gH. This selective killing activity resulted in strong suppression of infectious virus release from chronically infected cells induced into lytic phase. Moreover, the immunotoxin showed complementary activity with the herpesvirus DNA replication inhibitor ganciclovir; while more detailed studies will be required to distinguish between additivity versus formal synergy, the coordinate antiviral action of these distinct classes of agents speaks to their potential clinical value in combination.

The case for combination therapy with immunotoxin treatment plus replication inhibitors has been promoted for HIV-1, for which formal synergy between an anti-HIV targeted toxin and reverse transcriptase inhibitors was demonstrated (Ashorn et al., 1990), and combined treatment with an immunotoxin plus replication inhibitors in a murine model resulted in nearly complete suppression of viral rebound after cessation of treatment (Goldstein et al., 2000). These dramatic combination effects provided the basis for the proposal to test immunotoxin complementation of HAART as an approach to deplete persisting HIV infected cell reservoirs (Berger and Pastan, 2010). Parallels have been reported in the herpesvirus field; immunotoxins generated from mAbs against murine cytomegalovirus have been shown to display complementary activities with ganciclovir both in cell culture and in a murine model (Smee et al., 1996). These findings support continued investigation of immunotoxin treatment for pathologies associated with KSHV lytic phase infection.

Acknowledgments

We thank Deboeeta Chatterjee (NIAID, NIH) for helpful discussions and Virgilio Bundoc (NIAID, NIH) for excellent technical assistance. We extend our thanks to Joseph Newland (NIAID, NIH), Tapan Bera, John Weldon, Dawn Walker, and Ira Pastan (NCI, NIH) for technical advice and donation of PE-based immunotoxins. Jeffrey Vieira (U. Washington) kindly provided Vero-219 cells and the BacK-50 baculovirus. This research was funded in part by the Intramural Program of the NIH, NIAID. Y. C. and E. A. B. are co-inventors on an NIH-owned patent application on the YC15 hybridoma/mAb and the YC15-PE38 immunotoxin.

Abbreviations

KSHV	Kaposi’s sarcoma-associated herpesvirus
KS	Kaposi’s sarcoma
MCD	multicentric Castleman’s disease
PEL	primary effusion lymphoma
HAART	highly active antiretroviral therapy

mAb	monoclonal antibody
FBS	fetal bovine serum
scFv	single chain variable fragment
IPTG	isopropyl-1-thio-β-d-galactopyranoside

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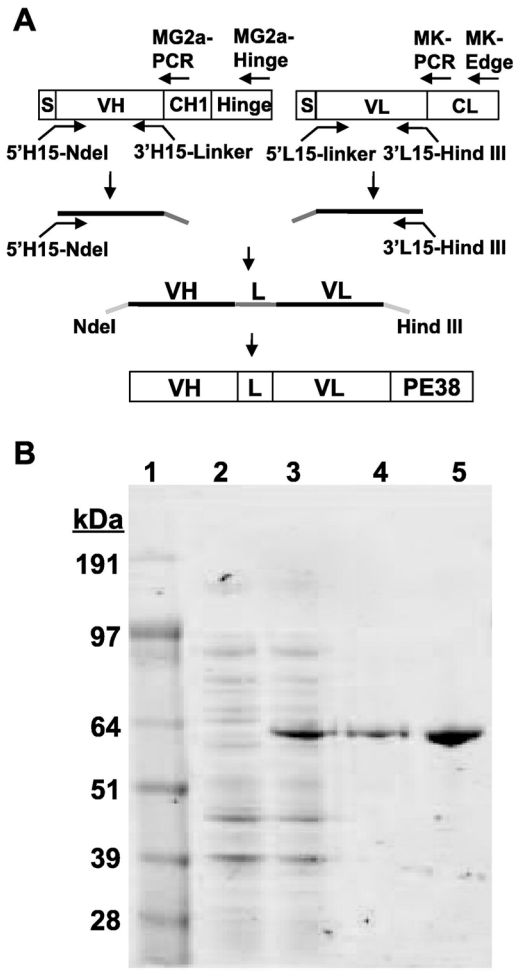

Fig. 1.

Expression and purification of recombinant YC15-PE38 immunotoxin direct against the KSHV gH glycoprotein (A) Schematic of construction of the YC15-PE38 immunotoxin expression plasmid and the relative positions of the PCR primers used for the amplification and cloning procedure (see Materials and Methods). The V_H and V_L segments of anti-KSHV gH mAb YC15 were linked by a 15 amino acid linker $(G_4S)_3$ and fused in frame to PE38. (B) SDS-PAGE analysis of steps in purification of YC15-PE38. Lane 1, Molecular weight markers (kDa values shown at the left). Lane 2, uninduced bacterial cell lysate. Lane 3, IPTG-induced bacterial cell lysate. Lane 4, inclusion body preparation from induced cells. Lane 5, purified YC15-PE38 immunotoxin. Similar results were obtained for YC15-$PE38^{E553D}$ containing the inactivating mutation in the PE38 moiety.

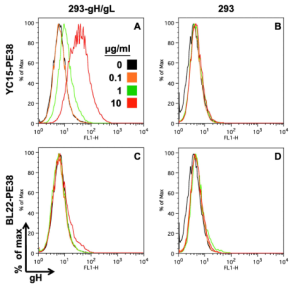

Fig. 2.

Flow cytometry analysis of the YC15-PE38 immunotoxin binding to KSHV gH-expressing transfectant cell line. YC15-PE38 and the control immunotoxin BL22-PE38 were labeled with Alexa fluor 488 and incubated at the indicated concentrations with the YC-293-gH/gL stable transfectant cell line or parental cells. After washing, the cellbound immunotoxins were detected by flow cytometry.

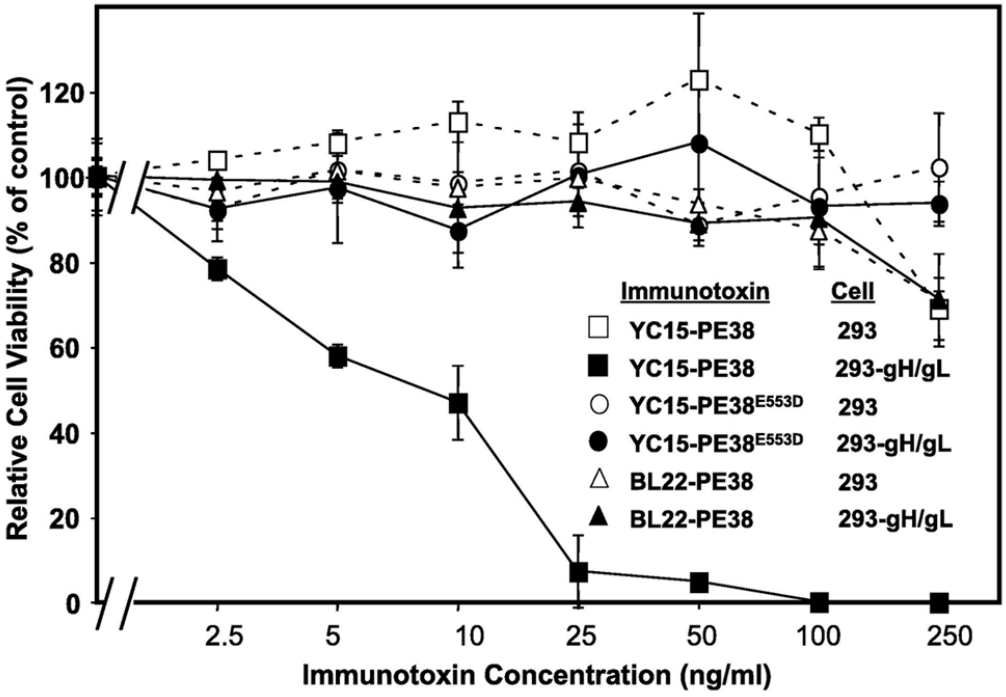

Fig. 3.

Direct cytotoxicity assay of YC15-PE38 against cell line expressing KSHV gH. 293 gH/gL stable transfectant cells (designated 293-gH/gL) or parental 293 cells were treated for 3 days with the indicated concentrations of YC15-PE38 or the negative control proteins YC15-PE38^{E553D} or BL22-PE38. Cell viability was measured by the WST-8 assay as describe in Materials and Methods. Data points indicate the means of triplicate samples; error bars represent standard deviations.

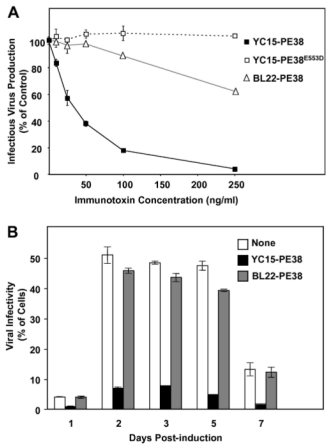

Fig. 4.

Effect of YC15-PE38 on infectious KSHV production from activated Vero-219 cells. (A) Vero-219 cells were activated by recombinant baculovirus BacK50 plus sodium butyrate, and treated with different concentration of YC15-PE38, YC15-PE38^{E553D} or control BL22-PE38 for 3 days. The supernatants were harvested and assayed for infection of fresh 293 cells to measure the infectious KSHV-r219 been produced from activated cells. The amount of infectious virus been produced from activated Vero-219 cells in the absence of immunotoxin was set as 100%. (B) Activated Vero-219 cells were treated with 100ng/ml of YC15-PE38 (black) or control BL22-PE38 (gray) or without immunotoxin (white). The infectious virus titer was measured at different time points post induction. Each data point represents the mean of triplicate culture wells, with error bars indicating standard deviations.

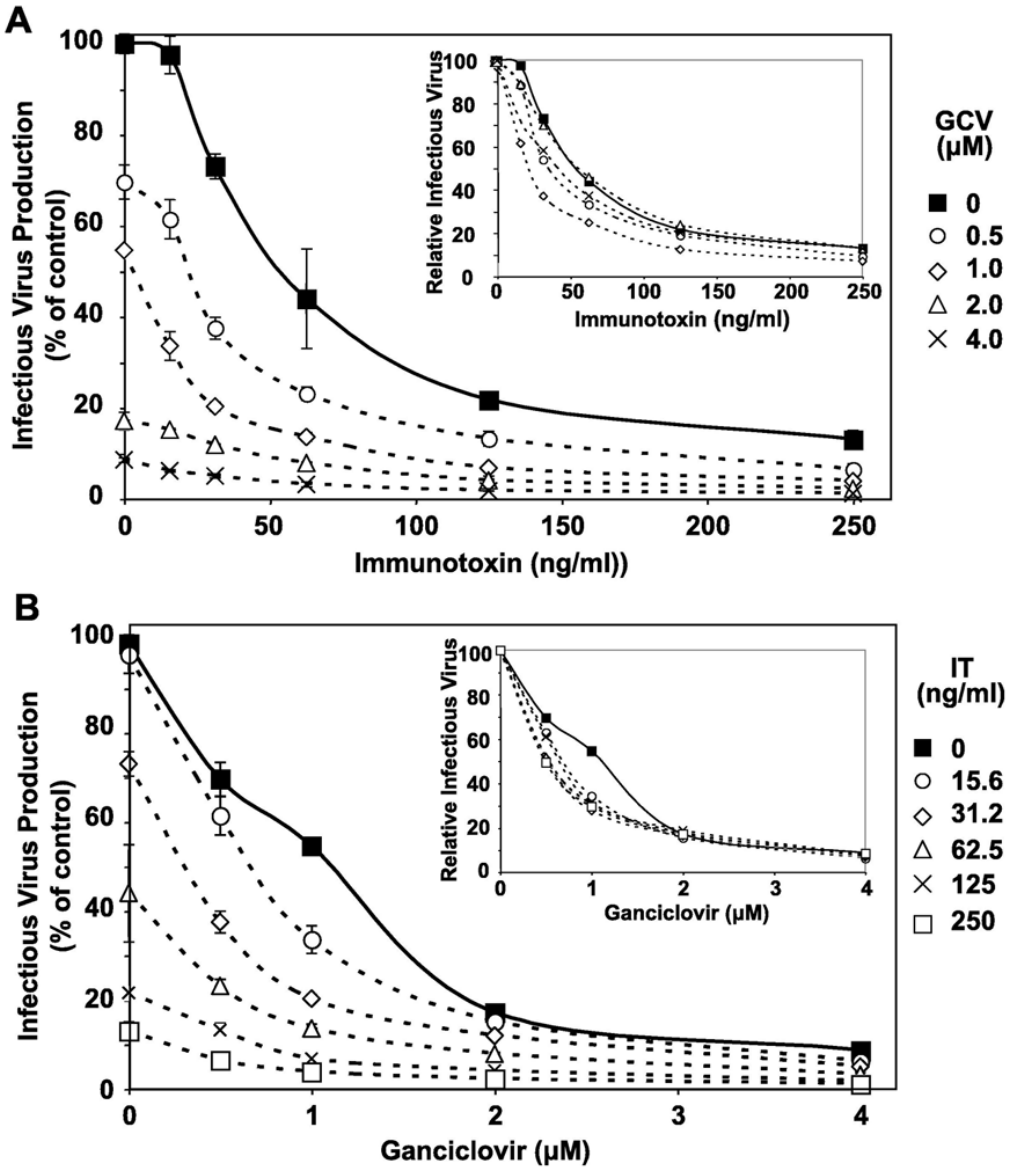

Fig. 5.

Effects of combination treatment with immunotoxin (IT) YC15-PE38 and ganciclovir on infectious KSHV production. Activated Vero-219 cells were treated for 3 days with combinations of YC15-PE38 and ganciclovir over the indicated concentration ranges for each agent. The amount of infectious virus produced was determined, with the value obtained in the absence of either agent set as 100%. Each data point represents the mean of triplicate culture wells, with error bars indicating standard deviation. A. Data are plotted as dose-response curves of YC15-PE38 in the presence of varying concentrations of ganciclovir; B. Data are plotted as dose-response curves of ganciclovir in the presence of varying concentrations of YC15-PE38. In each panel, the insets display the same data sets, with the values obtained in the absence of the agent on the X axis set as 100% for each dose-response curve.