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RNase inhibition of human immunodeficiency virus infection of H9 cells

(AIDS/eosinophil-derived neurotoxin)

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ABSTRACT **Onconase and bovine seminal RNase, two members of the RNase A superfamily, inhibit human immunodeficiency virus type 1 replication in H9 leukemia cells 90–99.9% over a 4-day incubation at concentrations not toxic to uninfected H9 cells. Two other members of the same protein family, bovine pancreatic RNase A and human eosinophil-derived neurotoxin, have no detectable antiviral activity, demonstrating a strikingly selective antiviral activity among homologous ribonucleases. The antiviral RNases do not appear to affect viral particles directly but inhibit replication in host cell cultures. Onconase, already in clinical trials for cancer therapy, and bovine seminal RNase have potential as antiviral therapeutics.**

Some cytotoxic proteins may play physiological roles in preventing viral replication. Pokeweed antiviral protein (PAP), for example, inhibits viral replication in plants (1, 2) and in mammalian cell culture (3, 4). The mechanism of viral inhibition stems from the enzymatic deglycosylation of the 28S rRNA by PAP, inhibiting protein synthesis and causing host cell death (5–7). In plants, PAP is safely sequestered outside of cells in the absence of virus. It has been proposed that PAP enters the cell cytosol during viral penetration, resulting in the death of infected cells and the prevention of viral reproduction (4, 8). In mammalian cell culture, PAP inhibits the replication of influenza virus (3) and poliovirus (4). Similar results have been found with plant proteins homologous to PAP, including abrin A chain (8), trichosanthin (9), and bryodin (10), that all appear to function like PAP. Diphtheria toxin and *Pseudomonas* exotoxin A, which enzymatically inactivate protein synthesis differently than the plant toxins, also become more toxic upon viral penetration of cells (11, 12).

Several members of the PAP family have been examined for anti-human immunodeficiency virus (HIV) activity. PAP, MAP 30, and TAP 29 block HIV replication at doses lower than those that are cytotoxic to cells in culture (13–15). Tricosanthin is another plant protein homologous to PAP that expresses *N*-glycosidase activity, inactivates protein synthesis, and has abortifacient activity. It inhibits HIV-1 infection in cultured cells (9) and has been tested in man for AIDS therapy (16, 17).

RNases may also play physiological roles in viral defenses. Viral infection causes RNase induction in certain plants (18) that may help block viral replication on plant leaves (19). In animals, interferon can trigger viral resistance by activating a 2-5A-dependent RNase (20) that may specifically degrade viral RNA.

RNases also can be toxic to cells (21, 22). Two human RNases homologous to RNase A, eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein, occur in eosinophil granules apparently to help mediate the cytotoxic activity of eosinophils, and these two RNases are very toxic to certain neurons (23). Onconase (Alfacell, Bloomfield, NJ), a frog RNase also homologous to RNase A, is toxic to mammalian cells in culture and expresses anticancer activity in animal models (24). It appears that Onconase binds to the cell surface, enters the cytosol, and degrades RNA to cause cell death (25). Another member of the RNase A superfamily, bovine seminal RNase (BS-RNase), also shows anti-cancer activity similar to that of Onconase *in vitro* and *in vivo* (26, 27). Although these RNases can be toxic to cells when applied to the outside of cells, RNases become thousands of times more toxic when artificially introduced into the cytosol (28, 29).

Entry into the cell cytosol appears to be the rate-limiting step for the toxicity of RNases (21), and viruses may be able to function in transporting cell surface or virus-bound RNases into the cytosol. Consistent with this model, several fungal RNases have been found to enter cells upon picornavirus, adenovirus, and Semliki Forest virus infection (8). At subtoxic concentrations of mammalian RNases, where the RNase binds the cell surface but fails to enter the cell cytosol, HIV virus particles may carry RNases into the cell where they may efficiently degrade viral and/or cellular RNA and inhibit viral replication. By studying four members of the RNase A superfamily we find that Onconase and BS-RNase, in contrast to two other homologous RNases, inhibit HIV-1 replication *in vitro* in H9 leukemia cells.

MATERIALS AND METHODS

RNases. Onconase was purified from frog eggs as reported (30) and BS-RNase was purified from bull semen as reported (31). RNase A was purchased from Calbiochem. Recombinant EDN was purified from bacteria expressing the synthetic gene for EDN (unpublished data).

HIV Infectivity and p24 Assays. Uninfected cells of the CD4-positive H9 lymphocyte line and HIV-1 persistently infected (HTLV-IIIB strain) H9 cells were obtained from Robert Gallo (National Cancer Institute, National Institutes of Health, Bethesda, MD). Both the uninfected and HIV-1 infected H9 cells were grown in RPMI 1640 medium (Mediatech, Herndon, VA) plus 10% heat-inactivated fetal bovine serum and 50 μg of gentamycin per ml (BioWhittaker).

The HIV-1 inoculum used in these studies was prepared by subdividing the persistently infected H9 cultures at a 1:4 split

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Abbreviations: HIV, human immunodeficiency virus; PAP, pokeweed antiviral protein; EDN, eosinophil-derived neurotoxin; BS-RNase, bovine seminal RNase; EIA, enzyme immunoassay.

ratio and incubating the cultures at 37°C in an atmosphere of 5% CO_2 in air. Cells and medium were harvested from these cultures 4 days later and the cells and the extraneous debris were removed by centrifugation at 400 × *g* for 10 min. The supernatant medium was removed from the cell pellet and filtered through a 0.45-μm-pore size membrane (Nalge). The HIV-1-containing filtrate was aliquoted, quick frozen in an alcohol-frozen CO_2 bath, and stored at −70°C. HIV-1 aliquots were only thawed once; experimentally unused portions were discarded.

HIV-1 infectivity was determined by endpoint titration in uninfected H9 cells. H9 cells were plated in 25-cm^2 tissue culture flasks (Costar) at 2 × 10^5 cells per ml, 5 ml of cells and medium per flask. Experimental samples were diluted in growth medium in serial 10-fold concentration increments, and duplicate H9 cultures were inoculated with 0.2 ml of diluted sample. On day 4 after inoculation, an additional 7 ml of growth medium was added to each culture. Beginning 7 days after inoculation and continued over a period of at least 21 days, cultures were subdivided 1:4 every 4–6 days. Cultures were observed microscopically for HIV-1-induced syncytia, and tentative 50% endpoints were determined. Infectivity titers computed from syncytia formation results were verified by HIV-1 p24 antigen capture enzyme immunoassay (EIA).

The concentrations of p24 antigen in experimental samples were also determined by quantitative HIV-1 p24 antigen capture EIA performed in accordance with instructions provided with the kits by the manufacturer (Coulter). Samples with p24 antigen concentrations exceeding that on the linear portion of the standard curve were diluted and concentrations were computed by multiplication of results by the dilution factor.

Cell Cytotoxicity Assays. H9 cells were plated into 96-well plates in RPMI 1640 medium containing 10% fetal calf serum and 50 μg of gentamycin per ml. Each well contained 100 μl of medium and 2 × 10^4 cells per well. RNases were added to the appropriate concentration in 11 μl of buffer. The cells were incubated for 1, 2, 3, or 4 days, pulsed with [^{14}C]leucine for 3 hr, harvested onto glass fiber filters in a PhD harvester, counted, and compared to control cultures lacking the RNases as described (25).

RESULTS AND DISCUSSION

We assayed the effect of Onconase, EDN, bovine pancreatic RNase A, and BS-RNase on H9 cells in culture. EDN and RNase A were nontoxic to H9 cells up to 10^{-6} M concentrations after 1, 2, 3, and 4 days in culture (Fig. 1). In contrast, Onconase inhibited cell protein synthesis at 10^{-6} M (Fig. 1*B*). BS-RNase inhibited protein synthesis in H9 cells to some extent at 10^{-6} M and more dramatically at 10^{-5} M (Fig. 1*D*). These results are similar to those seen in other cell types (25, 26, 32). All four RNases were nontoxic to H9 cells at 10^{-7} M and were assayed for anti-HIV activity at this concentration.

When H9 cells were infected with HIV-1, exponential amplification of viral p24 antigen and reverse transcriptase activity was seen (Fig. 2 and data not shown). The level of p24 antigen seen at day 0 was due to the residual HIV-1 inoculum remaining after H9 cells were washed following HIV-1 adsorption and penetration. Addition of 10^{-7} M EDN (Fig. 2*A*) or 10^{-7} M RNase A (Fig. 2*B*) to the cell cultures had no significant effect on viral replication. Onconase, however, inhibited p24 expression 99–99.9% at 10^{-7} M (Fig. 2 *A* and *B*). In these Onconase-treated cultures, a significant reduction of p24 antigen levels relative to control cultures was apparent 1

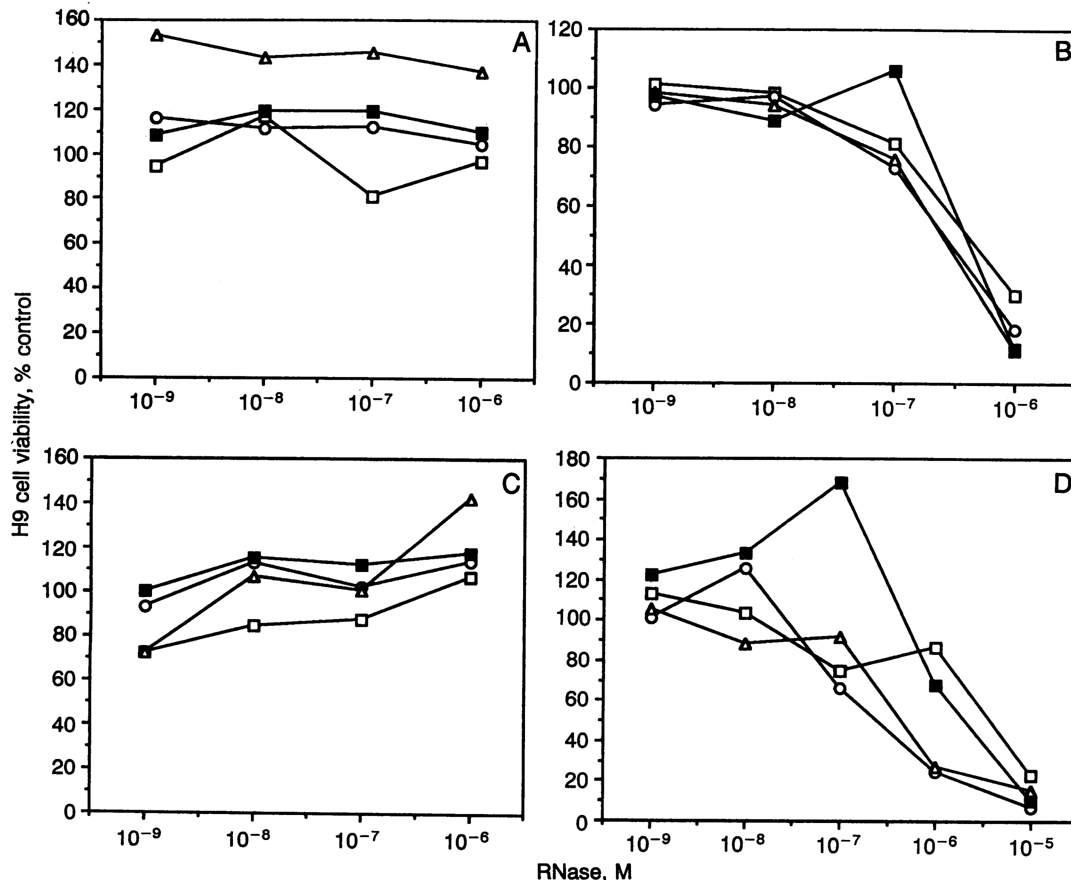

FIG. 1. H9 cell sensitivity to RNases. H9 cells were incubated 1 (□), 2 (○), 3 (△), or 4 (■) days with bovine pancreatic RNase A (*A*), Onconase (*B*), EDN (*C*), and BS-RNase (*D*).

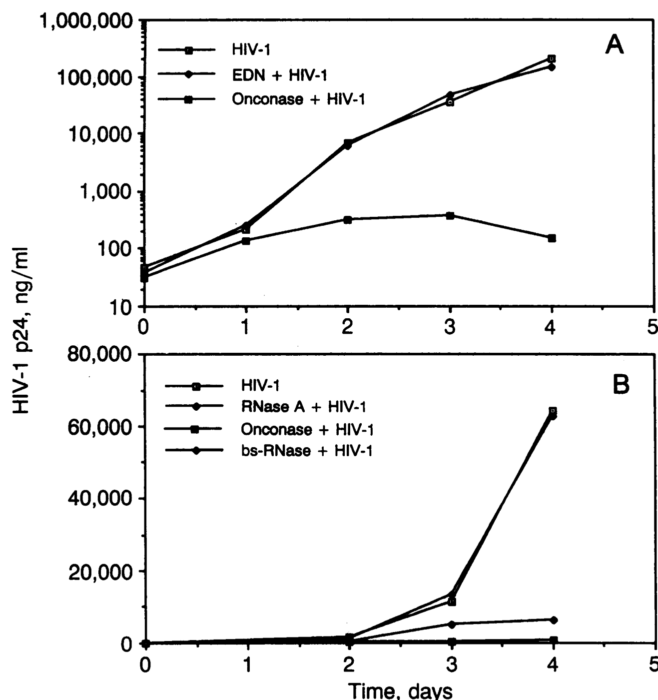

FIG. 2. HIV-1 replication in H9 cultures treated with 10^{-7} M Onconase, EDN, RNase A, and BS-RNase in two different experiments. The results of experiment A (*A*) are plotted on a logarithmic scale and the results of experiment B (*B*) are plotted on a linear scale. The HIV-1 inoculum was incubated with or without a 10^{-7} M concentration of the specified RNase or control medium for 1 hr at 37°C prior to adsorption to H9 cells (2×10^6 cells per ml) for 2 hr at 37°C. The 10^{-7} M concentration of the RNases was maintained throughout HIV-1 adsorption where appropriate. The cells were washed three times with 10 ml of RPMI 1640 medium to remove residual unadsorbed virus. The washed H9 cells were resuspended at 2×10^5 cells per ml in 5 ml of RPMI 1640 medium plus 10% fetal bovine serum and 50 μg of gentamycin per ml and incubated at 37°C in 5% CO_2 in air. Cultures were sampled daily up to 4 days following infection and monitored for p24 antigen by antigen capture EIA. Onconase at 10^{-7} M dramatically inhibited HIV-1 replication, whereas RNase A and EDN at 10^{-7} M had no effect on HIV-1 replication. BS-RNase at 10^{-7} M inhibited HIV-1 replication but to a lesser degree than Onconase.

day after infection and only minimal increases over background were detected over the 4-day incubation period. We compared the antiviral activity of BS-RNase to the homologues, RNase A, EDN, and Onconase. BS-RNase significantly inhibited HIV-1 replication in H9 cells (Fig. 2*B*) at 10^{-7} M, a concentration that did not inhibit uninfected H9 cell protein synthesis (Fig. 1*D*). The inhibition, however, was reproducibly smaller than that of Onconase and larger than that of EDN or RNase A.

HIV-1 infection of H9 cells causes the formation of large syncytial cell aggregates (Fig. 3*B*). Syncytium formation was almost totally abrogated in HIV-1-infected H9 cells incubated in medium containing 10^{-7} M Onconase (Fig. 3*C*) or 10^{-7} M BS-RNase (Fig. 3*D*). The cell aggregates in Onconase- and BS-RNase-treated H9 cells inoculated with HIV-1 (Fig. 3 *C* and *D*) resembled H9 cells not infected with HIV-1 (Fig. 3*A*). In contrast, HIV-1-infected cultures treated with RNase A or EDN, RNases that did not inhibit HIV-1 replication, showed syncytia indistinguishable from those seen in HIV-1-infected control cultures (data not shown). Thus, reduced syncytium formation correlates directly with the capacity of specific RNases to inhibit HIV-1 replication measured by p24 antigen levels (Fig. 2).

The time course of inhibition shows that Onconase prevents the logarithmic growth of HIV (Fig. 2). Ten-fold less Onconase, 10^{-8} M, also inhibited HIV-1 replication although to a lesser degree than 10^{-7} M Onconase (Fig. 4). By 4 days, 10^{-8} M Onconase inhibited viral replication 90%. Therefore, after 4 days, Onconase inhibited HIV replication at doses 1/100th those that inhibited cell viability (Fig. 1). BS-RNase also inhibited HIV-1 infection at 10^{-8} M (data not shown).

Onconase is less active in RNA degradation than is RNase A or EDN (30). We examined whether or not RNase enzyme activity of Onconase is required for antiviral activity. Active site histidine residues of Onconase can be alkylated with iodoacetamide to decrease enzyme activity, analogous to histidine modifications of RNase A (30). Onconase alkylated with different amounts of iodoacetamide to yield preparations of alkylated Onconase with 30% and 2% RNase activity relative to that of native Onconase were compared in the HIV-1 assay performed as in Fig. 2. Whereas native Onconase inhibited HIV-1 replication, alkylated Onconase with 2% RNase activity did not inhibit HIV-1 replication at 10^{-7} M. The alkylated Onconase with 30% enzyme activity appeared intermediate between the native and the 2% active alkylated Onconase (data not shown). Thus, RNase activity of Onconase appears necessary for the antiviral activity.

We examined whether Onconase exerted its inhibitory effect on HIV-1 p24 antigen production and syncytium formation by degrading the RNA genome of HIV-1 virions prior to their uptake by susceptible cells, thus reducing their infectivity. HIV-1 was incubated with or without 10^{-7} M or 10^{-8} M Onconase, in the same culture medium and under the same conditions as in the other experiments. Samples of Onconase-treated HIV-1 and untreated control HIV-1 were taken after a 2-hr incubation period at 37°C and titrated in H9 cells for infectivity as described in *Materials and Methods*. As shown in Table 1, no significant differences were found due to Onconase treatment of the HIV-1 virions. Thus, the inhibitory effects of Onconase appear to be mediated at the cellular level rather than by a direct effect of the RNase on the infectivity of the virions. However, our results suggest that treatment of uninfected H9 cells with 10^{-7} M Onconase only minimally reduces protein synthesis (Fig. 1). Thus, HIV-1 infection of susceptible mammalian cells may impart increased toxicity of certain RNases to the host cell.

There is surprising specificity in HIV-1 inhibition among the four homologous RNases examined, and the structural basis for the dramatic difference between the two RNases inactive against HIV (RNase A and EDN) and the two active RNases (Onconase and BS-RNase) is unclear. EDN may play host defense roles in eosinophil killing of parasites and RNase A is thought to function in digestion. The physiological roles of Onconase and BS-RNase are unknown. The potency of cell cytotoxicity of the RNases correlates with anti-HIV activity. Although all four RNases were tolerated by H9 cells at 10^{-7} M, the two antiviral RNases were both toxic at 10^{-6} M, whereas the other two RNases were not. However, the structural basis of the cellular cytotoxicity of Onconase and BS-RNase remains unknown (21, 22). The RNases examined, RNase A, EDN, BS-RNase, and Onconase, differ in ribonucleolytic activity by several orders of magnitude. Although RNase activity is required for the antiviral activity of Onconase, for the cytotoxicity of Onconase (25) and for the neurotoxicity of EDN (33) and Onconase (34), enzyme activity *per se* does not correlate with potency of RNase cytotoxicity, neurotoxicity, or antiviral activity. Structurally, Onconase is about 30% identical in amino acid sequence to RNase A and EDN (30), and BS-RNase subunit sequence (35) is even closer to that of RNase A with >80% identity. The three-dimensional structure of BS-RNase (36) and that of Onconase (37) are very similar to that of RNase A. BS-RNase, however, differs in tertiary structure from Onconase and the other RNases as it is a disulfide-linked homodimer (38). Further structure/function studies based upon the

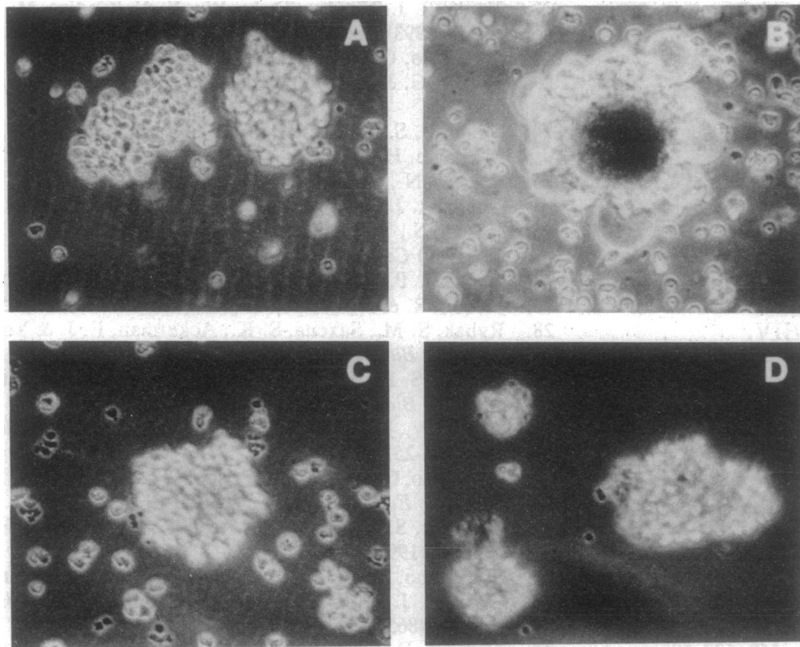

FIG. 3. Failure of HIV-1 to induce syncytia in H9 cells in the presence of Onconase and BS-RNase. H9 cell aggregates are shown after 4 days in the absence of HIV-1 (*A*), in the presence of HIV-1 (*B*), in the presence of HIV-1 and 10^{-7} M Onconase (*C*), and in the presence of HIV-1 and 10^{-7} M BS-RNase (*D*). Syncytium formation is dramatic in the presence of HIV-1 alone (*B*) and this is prevented by Onconase and BS-RNase (*C* and *D*). The H9 cell aggregates in *C* and *D* have essentially the same morphology as those in uninfected H9 cell cultures (*A*). (×20.)

three-dimensional structure of these RNases may soon reveal the domains of Onconase and BS-RNase involved in anticancer and antiviral activities and yield insight into the cellular mechanisms of toxicity. Perhaps insensitivity to placental RNase inhibitor or binding the target cell surface correlates with the degree of cellular toxicity and anti-HIV activity (25, 39, 40).

EDN and Onconase are both toxic to Purkinje cells, whereas RNase A is 5000 times less active (34). However, EDN and RNase A both lack antiviral activity against HIV-1. Therefore, the degree of neurotoxicity does not correlate with the anti-HIV activity among these homologous RNases.

In view of the recently established mechanism of action of Onconase whereby the cytotoxicity against the 9L glioma cell line appeared to be due to enzymatic degradation of cytosolic RNA, possibly rRNA, and the proposed mechanism of a series of antiviral plant proteins being that of ribosome inactivation, we suggest that the specific viral sensitivity to RNases may be due to virus facilitating the transport of Onconase and BS-RNase into the cytosol where they become toxic to the host cell. Although Onconase was not found to have effects on HIV-1 virus particles, at this point it is not known whether or not Onconase and BS-RNase demonstrate

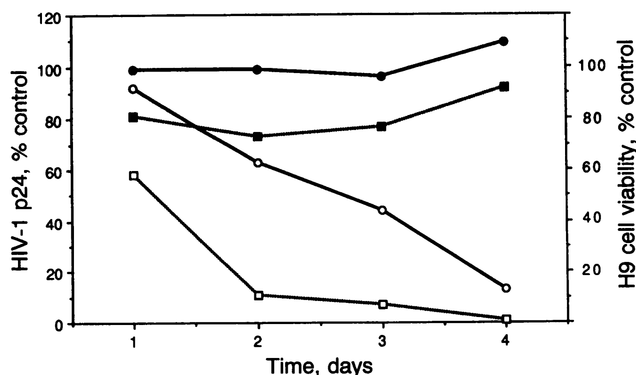

FIG. 4. Dose–response of Onconase on HIV-1 replication and on H9 cell protein synthesis. HIV-1 p24 antigen levels are indicated in the presence of 10^{-7} M Onconase (□) and 10^{-8} M Onconase (○). Cell protein synthesis is shown in uninfected H9 cells in the presence of 10^{-7} M Onconase (■) and 10^{-8} M Onconase (●). Onconase inhibits HIV-1 replication and not H9 cell viability.

direct anti-HIV activity, including the potential to cleave the RNA of HIV-1 intracellularly. However, it appears in our experiments that the RNases primarily exert their cytotoxic activity by way of the HIV-infected mammalian cell rather than on the virus particle itself.

A number of toxic proteins have been engineered to bind selectively to HIV-infected cells. Monoclonal antibodies or CD4 molecules linked to ricin A chain (41, 42), *Pseudomonas* exotoxin A (43, 44), and PAP (13, 45) inhibit viral replication in cell culture and several of these proteins are currently in human trials. Possibly the anti-HIV activity of Onconase can be further augmented by engineering cell-type-specific binding moieties into the Onconase molecule. Onconase (46) and RNase A (28, 47) have been coupled to monoclonal antibodies to target tumor cells and it will be important to determine whether CD4 or antibodies that bind HIV-infected cells will increase the endogenous antiviral activity of Onconase.

Onconase has already been tested clinically for cancer and appears to be well tolerated (48), although blood levels of Onconase have not yet been determined. When this is known, comparison of the pharmacology and the maximal tolerated doses of Onconase in patients with the time course and dose dependency of antiviral activity in culture may help predict the extent of antiviral activity possible in man. Antiviral RNases in general and Onconase and BS-RNase in particular should be tested *in vivo* for antiviral activity.

Plant ribosome-inactivating proteins alone and conjugated to monoclonal antibodies are undergoing testing for anti-HIV activity in man and bacterial toxins fused to CD4 have also

Table 1. Effect of Onconase on HIV-1 virions

Treatment	Preincubation time, hr	HIV-1 infectivity
HIV-1	1	$10^{5.2}$
	2	$10^{5.2}$
+ 10^{-8} M Onconase	1	$10^{5.2}$
	2	$10^{5.2}$
+ 10^{-7} M Onconase	1	$10^{5.2}$
	2	$10^{4.95}$

HIV-1 virions were preincubated with Onconase at the prescribed concentrations and times at 37°C. The HIV-1 infectivity or viral titer was then determined by serial dilutions of the viral samples onto H9 cells. The viral titer was not significantly affected by the preincubation with Onconase.

been tested clinically. These plant and bacterial toxins may be poorly tolerated in man and are quite immunogenic. In contrast, Onconase and BS-RNase are members of a superfamily containing several human proteins, including pancreatic RNase, EDN, eosinophil cationic protein, and angiogenin (21, 22). Thus these RNase homologues of human proteins may be safer and recognized as less foreign and less immunogenic than plant and bacterial toxins. Further understanding of the structure/function relationships that cause certain members of this superfamily to exert antiviral activity may allow these features to be engineered into the human members of this superfamily, bringing us even closer to the humanization of proteins for treatment of HIV.

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1. Wyatt, S. & Shepherd, R. (1969) *Phytopathology* **59**, 1787–1794.
2. Lodge, J. K., Kaniewski, W. K. & Tumer, N. E. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7089–7093.
3. Tomlinson, J., Walker, V., Flewett, T. & Barclay, G. (1974) *J. Gen. Virol.* **22**, 225–232.
4. Lee, T., Crowell, M., Shearer, M. H., Aron, G. M. & Irvin, J. D. (1990) *Antimicrob. Agents Chemother.* **34**, 2034–2037.
5. Irvin, J. D. (1975) *Arch. Biochem. Biophys.* **169**, 522–528.
6. Irvin, J. D. & Aron, G. M. (1982) *FEBS Lett.* **148**, 127–130.
7. Endo, Y., Tsurugi, K. & Lambert, J. (1988) *Biochem. Biophys. Res. Commun.* **150**, 1032–1036.
8. Fernandez-Puentes, C. & Carrasco, L. (1980) *Cell* **20**, 769–775.
9. McGrath, M., Huang, K., Caldwell, S., Gaston, I., Luk, K.-C., Wu, P., Ng, V., Crowe, S., Danials, J., Marsh, J., Deinhart, T., Lekas, P., Vennary, I., Yeung, H.-W. & Lifson, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2844–2848.
10. Wachinger, M., Samtleben, R., Gerhauser, C., Wagner, H. & Erfle, V. (1993) *Res. Exp. Med.* **193**, 1–12.
11. Yamaizumi, M., Uchida, T. & Okada, Y. (1979) *Virology* **95**, 218–221.
12. FitzGerald, D., Padmanabhan, R., Pastan, I. & Willingham, M. (1983) *Cell* **32**, 607–617.
13. Zarling, J. M., Moran, P. A., Haffar, O., Sias, J., Richman, D. D., Spina, C. A., Myers, D. E., Kuebelbeck, V., Ledbetter, J. A. & Uckun, F. M. (1990) *Nature (London)* **347**, 92–95.
14. Lee-Huang, S., Huang, P., Nara, P., Chen, H.-C., Kung, H.-F., Huang, P., Huang, H. & Huang, P. (1990) *FEBS Lett.* **272**, 12–18.
15. Lee-Huang, S., Huang, P., Kung, H.-F., Li, B.-Q., Huang, P., Huang, P., Huang, H. & Chen, H.-C. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6570–6574.
16. Kahn, J., Kaplan, L., Gambertoglio, J., Bredesen, D., Arri, C., Turin, L., Kibort, T., Williams, R., Lifson, J. & Volberding, P. (1990) *AIDS* **4**, 1197–1204.
17. Byers, V., Levin, A., Waites, L., Starrett, B., Mayer, R., Clegg, J., Price, M., Robins, R., Delaney, M. & Baldwin, R. (1990) *AIDS* **4**, 1189–1196.
18. Chakravorty, A. K., Shaw, M. & Scrubb, L. A. (1974) *Nature (London)* **247**, 577–580.
19. Loring, H. (1941) *J. Gen. Physiol.* **25**, 497–505.
20. Zhou, A., Hassel, B. A. & Silverman, R. H. (1993) *Cell* **72**, 753–765.
21. Youle, R. J., Newton, D. L., Wu, Y.-N., Gadina, M. & Rybak, S. M. (1993) *Crit. Rev. Ther. Drug Carrier Syst.* **10**, 1–28.
22. D'Alessio, G. (1993) *Trends Cell Biol.* **3**, 106–109.
23. Gleich, G. J. & Adolphson, C. R. (1986) *Adv. Immunol.* **39**, 177–253.
24. Mikulski, S. M., Ardelt, W., Shogen, K., Bernstein, E. H. & Menduke, H. (1990) *J. Natl. Cancer Inst.* **82**, 151–152.
25. Wu, Y.-N., Mikulski, S. M., Ardelt, W., Rybak, S. M. & Youle, R. J. (1993) *J. Biol. Chem.* **268**, 10686–10693.
26. Vescia, S., Tramontano, D., Augusti-Tocco, G. & D'Alessio, G. (1980) *Cancer Res.* **40**, 3740–3744.
27. Laccetti, P., Portella, G., Mastronicola, M. R., Russo, A., Piccoli, R. & D'Alessio, G. (1992) *Cancer Res.* **52**, 4582–4586.
28. Rybak, S. M., Saxena, S. K., Ackerman, E. J. & Youle, R. J. (1991) *J. Biol. Chem.* **266**, 21202–21207.
29. Saxena, S. K., Rybak, S. M., Youle, R. J. & Ackerman, E. J. (1991) *J. Biol. Chem.* **266**, 21208–21214.
30. Ardelt, W., Mikulski, S. M. & Shogen, K. (1991) *J. Biol. Chem.* **266**, 245–251.
31. D'Alessio, G., Floridi, A., Prisco, R. D., Pignero, A. & Leone, E. (1972) *Eur. J. Biochem.* **26**, 153–161.
32. Mikulski, S. M., Viera, A., Ardelt, W., Menduke, H. & Shogen, K. (1990) *Cell Tissue Kinet.* **23**, 237–246.
33. Sorrentino, S., Glitz, D. G., Hamann, K. J., Loegering, D. A., Checkel, J. L. & Gleich, G. J. (1992) *J. Biol. Chem.* **267**, 14859–14865.
34. Newton, D. L., Walbridge, S., Mikulski, S. M., Ardelt, W., Shogen, K., Ackerman, S. J., Rybak, S. M. & Youle, R. J. (1994) *J. Neurosci.* **14**, 538–544.
35. Suzuki, H., Parente, A., Farina, B., Greco, L., Montagne, R. L. & Leone, E. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 1305–1312.
36. Mazzarella, L., Capasso, S., Demasi, D., Lorenzo, G. D., Mattia, C. A. & Zagari, A. (1993) *Acta Crystallogr.* **49**, 389–402.
37. Mosimann, S. C., Johns, K. L., Ardelt, W., Mikulski, S. M., Shogen, K. & James, M. N. G. (1992) *Proteins Struct. Funct. Genet.* **14**, 392–400.
38. D'Alessio, G., DiDonato, A., Parente, A. & Piccoli, R. (1991) *Trends Biochem. Sci.* **16**, 104–106.
39. Blackburn, P. & Gavilanes, J. G. (1980) *J. Biol. Chem.* **255**, 10959–10965.
40. Murthy, B. S. & Sidershmukh, R. (1992) *Biochem. J.* **281**, 343–348.
41. Till, M., Ghetie, V., Gregory, T., Patzer, E., Porter, J., Uhr, J., Capon, D. & Vitetta, E. (1988) *Science* **242**, 1166–1168.
42. Pincus, S. H., Wehrly, K. & Chesebro, B. (1989) *J. Immunol.* **142**, 3070–3075.
43. Chaudhary, V., Mizukami, T., Fuerst, T., FitzGerald, D., Moss, B., Pastan, I. & Berger, E. (1988) *Nature (London)* **355**, 369–372.
44. Tsubota, H., Winkler, G., Meade, H., Jakubowski, A., Thomas, D. & Letvin, N. (1990) *J. Clin. Invest.* **86**, 1684–1689.
45. Kim, Y.-W., Fung, M., Sun, N.-C., Sun, C., Chang, N. & Chang, T. (1990) *J. Immunol.* **144**, 1257–1262.
46. Rybak, S. M., Newton, D. L., Mikulski, S. M., Viera, A. & Youle, R. J. (1993) *Drug Targeting Delivery* **1**, 3–10.
47. Newton, D. L., Ilercil, O., Laske, D. W., Oldfield, E., Rybak, S. M. & Youle, R. J. (1992) *J. Biol. Chem.* **267**, 19572–19578.
48. Mikulski, S. M., Grossman, A. M., Carter, P. W., Shogen, K. & Costanzi, J. J. (1993) *Int. J. Oncol.* **3**, 57–64.