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Mini review

Therapeutic potential of anti-HIV immunotoxins

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Abstract

In vitro analyses have shown anti-HIV immunotoxins to be among the most effective AIDS antivirals tested. Because HIV has been continually selected by antibody, immunotoxins targeted to constant domains of viral antigens may not elicit drug-resistant mutants. A clinical trial with CD4-PE40, a possibly flawed immunotoxin with nonspecific toxicity and short serum half-life, has reduced interest in this form of therapy. It is proposed that the use of an immunotoxin directed against gp41 in combination with a CD4-Ig chimera is more likely to have a therapeutic effect than CD4-PE40. Clinical trials are also underway utilizing an immunotoxin that eliminates activated T-cells, an important cellular locus of HIV-replication.

Keywords: HIV; AIDS; Anti-HIV immunotoxins; Immunotoxin

1. Introduction

The treatment of AIDS and HIV infection has three major goals: antiviral therapy aimed at eliminating HIV, the prevention and treatment of opportunistic infections and malignancies associated with AIDS, and immune restoration to correct the damage to the immune system caused by HIV. This review is concerned with the first aspect of AIDS therapy, the development of anti-HIV drugs. Two classes of HIV-specific antivirals are already approved for use: reverse transcriptase and protease inhibitors, and combinations of these drugs appear more efficacious than when each is used individually (Collier et al., 1996). Despite these advances, there are still significant limitations to existing anti-HIV therapies, especially the evolution of drug-resistant variants. It is therefore essential that the quest for new antivirals continue, particularly for agents with different modes of action and which may eliminate viral reservoirs not eradicated by other agents.

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Immunotoxins are bifunctional molecules, consisting of a targeting portion and the toxic moiety, generally either a plant or bacterial toxin (Pincus and Tolstikov, 1995). Immunotoxins are part of a broader class of agents termed immunoconjugates, in which other agents, such as radionuclides or cytotoxic drugs, are coupled to the targeting moiety. These agents have been used effectively and with little toxicity in the treatment of malignancies, graft rejection, and autoimmune diseases (Kaminski et al., 1993; Press et al., 1993; Strand et al., 1993; Frankel et al., 1996; Thrush et al., 1996).

The object of using immunotoxins to treat AIDS is to eliminate HIV-infected cells that are actively secreting virus and spreading infection. Immunotoxins may be targeted either to viral antigens on the surface of HIV-infected cells (referred to as anti-HIV immunotoxins) or to cellular molecules that define the cell type(s) in which HIV replicates. In vitro analyses demonstrate that anti-HIV immunotoxins are highly effective in eliminating infected cells and inhibiting the spread of infection through a cell culture. Significant enhancement of anti-HIV activity has been shown to occur when immunotoxins are used in combination with other antivirals, soluble CD4, or agents that inhibit the intracellular degradation of immunotoxins.

A clinical trial has been performed utilizing a chimeric CD4-toxin molecule. Little evidence of anti-HIV efficacy was demonstrated. However, this immunotoxin had significant drawbacks that may have limited its therapeutic utility. Because of this failure, interest in the use of anti-HIV immunotoxins has diminished. On the basis of in vitro data, nonspecific toxicity, and pharmacokinetics, antibody-based anti-gp41 immunotoxins used in combination with CD4-Ig chimeras have a much higher likelihood of success. Immunotoxins should not be abandoned as a possible treatment for HIV infection. Potential advantages include a different mode of action than existing antivirals, decreased likelihood of inducing infectious drug-resistant variants, and a high therapeutic index.

2. Immunotoxin structure and design

An immunotoxin functions by binding to the target cell, becoming internalized, and exerting a toxic action which kills the cell. Immunotoxins consist of three portions: the targeting moiety, the toxic substance, and a linker joining the two. Alternatively, the immunotoxin may be a chimeric protein in which the targeting and toxic portions are combined into a single molecule using recombinant DNA technology. The majority of this review is devoted to targeting moiety. The following paragraphs in this section will be devoted to a brief discussion of toxins and to linkers. A more thorough review of these subjects can be found elsewhere (Pincus and Tolstikov, 1995; Thrush et al., 1996).

A variety of plant and animal toxins, as well as cytotoxic drugs and radionuclides, have been used to kill cells targeted by immunotoxins and immunoconjugates. The choice of toxic moiety can be critical in the success of the immunotoxin because nonspecific, dose-limiting toxicities are often a function of this portion of the molecule. The most commonly used toxins, including ricin, pseudomonas exotoxin A, and diphtheria toxin, must be stripped from their native targeting moiety that delivers them to a large array of cells. This may be accomplished chemically or by genetic modification. Each of these toxins inhibits protein synthesis, either by destroying the structure of the ribosome (ricin A chain) or by inhibiting key synthetic factors (pseudomonas exotoxin A and diphtheria toxin). A number of other toxins have been identified and isolated, and novel chimeric toxins are being constructed utilizing genetic engineering techniques. Each are undergoing clinical testing in the form of immunotoxins in the hope of identifying the 'ideal' toxin, having little nonspecific toxicity, great efficacy against the target cell, and little immunogenicity.

The linker is responsible for maintaining the attachment of the targeting and toxic portions of the immunotoxin while in the circulation, but must be cleaved once the molecule is internalized within a cell. The linker may be omitted if the immunotoxin is a chimeric protein and the

toxin can either function within the chimeric protein or be freed by cleavage with a cellular protease. Most linkers have been designed to come apart under either acid or reducing conditions, which exist within the phagolysosome, the first cellular compartment encountered by the immunotoxin once it has been internalized into the target cell.

Once free from the targeting moiety, the toxin must be transported to the cellular site of its action, the cytoplasm for inhibitors of protein synthesis or the nucleus for cytotoxic drugs. Because a major function of the phagolysosome is the elimination of internalized materials, most of the immunotoxin internalized into a cell is destroyed by proteolytic digestion. Agents that inhibit lysosomal function, such as chloroquine, monensin, NH_4Cl, brefeldin A, or bafilomycin A1, have been shown to enhance the activity of immunotoxins.

3. Immunotoxins targeted to HIV antigens

The only HIV antigens that are expressed intact on infected cells and recognizable by antibodies are the envelope glycoproteins gp120 (extracellular) and gp41 (transmembrane). Although there is a high degree of inter-isolate sequence variability, there are also highly conserved constant regions in both gp120 and gp41. A number of human and murine monoclonal antibodies have been made against these epitopes. The well conserved CD4-binding region can also be targeted with CD4 itself. Many of the conserved regions are surface exposed on infected cells and on virions. A number of different immunotoxins directed against these regions have been made. The most extensively tested include a series of anti-gp120 and anti-gp41 Mabs conjugated to ricin A chain, made in our laboratory (Pincus et al., 1991, 1996; Pincus and McClure, 1993; Pincus and Tolstikov, 1995) and the chimeric immunotoxin containing CD4 attached to the toxic portion of pseudomonas exotoxin A, CD4-PE40 (Chaudhary et al., 1988; Ashorn et al., 1990, 1991; Kennedy et al., 1993).

Anti-HIV immunotoxins have been tested in a variety of different cell culture systems including T cell lines acutely or persistently infected with laboratory isolates of HIV, PHA blast cultures infected with either laboratory or primary clinical isolates, and macrophages. The immunotoxins were highly effective in killing infected cells and halting the spread of infection at therapeutically obtainable concentrations. Cytotoxic action was rapid (< 1 h) and was accompanied by an immediate cessation in the secretion of infectious virions (Pincus et al., 1989). Fig. 1 shows the effect of anti-HIV immunotoxins on infected cells. One report has indicated complete elimination of HIV from a cell culture with a combination of an immunotoxin and reverse transcriptase inhibitors (Ashorn et al., 1990) although others have found that the immunotoxin must be continually present to completely suppress HIV production (Tsubota et al., 1990; Winters and Merigan, 1993). We have found cytotoxicity of immunotoxins on HIV-infected cells at concentrations lower than 0.01 μg/ml with no non-specific toxicity on uninfected cells at 100 μg/ml (Pincus and McClure, 1993), an in vitro therapeutic index of greater than 10 000, far greater than that reported for any other anti-HIV drug.

Several maneuvers have been demonstrated to enhance the efficacy of anti-HIV immunotoxins. These include combining immunotoxins with other anti-retrovirals (Ashorn et al., 1990; Pincus and Wehrly, 1990), using drugs that inhibit the lysosomal degradation of immunotoxins, (Till et al., 1989; Pincus et al., 1994, 1996), and by the addition of soluble CD4 (Pincus and McClure, 1993; Pincus et al., 1996). CD4-mediated enhancement of immunotoxin action is restricted to anti-gp41 immunotoxins, but has been seen with immunotoxins directed against at least four different, well conserved epitopes on gp41. The addition of CD4 lowers the concentration of immunotoxin required for cytotoxicity 30–100-fold without increasing nonspecific toxicity. Enhancement occurs at concentrations of CD4 that are readily obtainable in human tissues and is the result of two different effects: increased surface exposure of gp41 epitopes and increased rates of

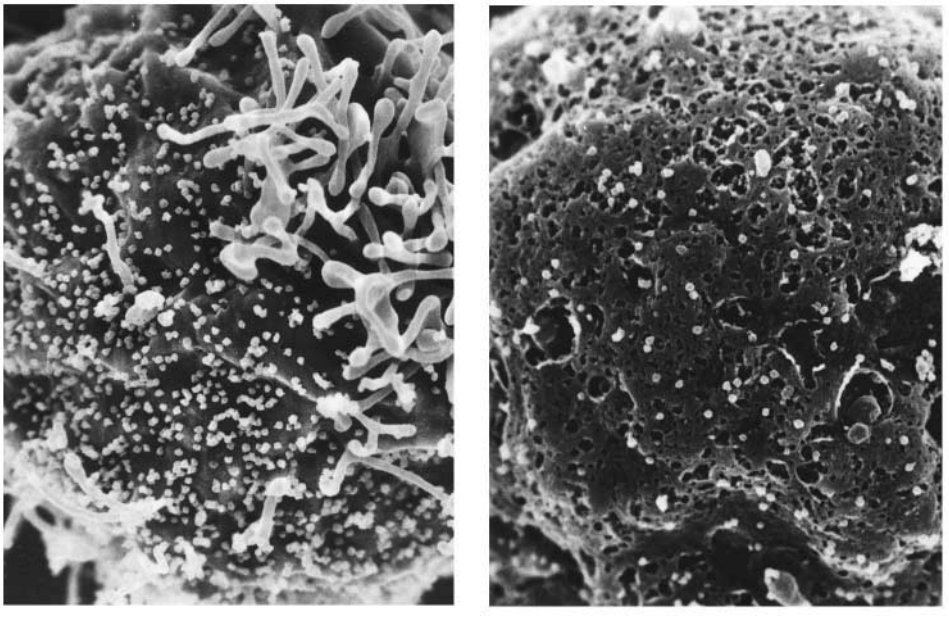


Fig. 1. Immunotoxin killing of HIV-infected cells. Scanning electron micrographs of an untreated HIV-infected cell (left) and a cell four hours after immunotoxin treatment (right). The cells are persistently HIV-infected H9/NL4-3 cells (Pincus and Wehrly, 1990). The untreated cell contains many budding virions over the whole surface, as well as the much larger cellular projections that are also seen on uninfected cells. Following immunotoxin treatment, massive disruptions of the cell membrane and loss of cellular projections are observed. Budding virions are still seen, but experiments have shown that these are non-infectious and probably result from contraction of the dying cell's membrane over already assembled core proteins. Scanning electron microscopy by David Dorward, NIAID Rocky Mountain Laboratories.

internalization from the cell surface. CD4-mediated immunotoxin enhancement occurs even in primary clinical isolates that are resistant to neutralization by soluble CD4 (Pincus et al., 1996).

The cytotoxic effect of immunotoxins directed against the HIV-envelope proteins reveals an important aspect of the cell biology of HIV infection (Pincus et al., 1994). For an immunotoxin to kill a cell, it must be internalized. Many virologists consider cell surface gp120 and gp41 solely as a manifestation of viral budding. Yet the ability of immunotoxins to kill HIV-infected cells indicates that a portion of cell surface gp120/gp41 recirculates, because the immunotoxin must be internalized to kill the cell. Moreover, the ability of CD4 to enhance the rate of internalization indicates that the viral envelope functions in a manner similar to a cellular receptor, showing increased rates of internalization in the presence of ligand (Pincus and McClure, 1993).

For a number of reasons gp41 is likely to be a better target of immunotoxin therapy than gp120 (Pincus, 1994). Gp41 is an integral transmembrane protein, whereas gp120 is non-covalently attached to gp41. Soluble gp120 can be released from the target cell and inhibit the binding of anti-gp120 immunotoxins. This is particularly true in the presence of CD4. But most importantly, the activity of anti-gp41 immunotoxins can be markedly enhanced in the presence of soluble CD4 so that they are 30–100 times more effective than anti-gp120.

4. Immunotoxins directed against the IL-2 receptor or other cellular markers

An alternative approach to deliver immunotoxins to HIV-infected cells is to use cell surface markers that define the subset of cells in which

HIV replicates. The IL-2 receptor defines a subset of T cells that are in an activated state. The IL-2 receptor may be targeted with monoclonal antibodies (anti-CD25) or with IL-2 itself. Studies with immunotoxins have clearly demonstrated that in cultures of peripheral blood mononuclear cells, cells expressing the IL-2 receptor are the primary, if not sole, site of HIV replication (Finberg et al., 1991; Bell et al., 1993; Ramilo et al., 1993; Borvak et al., 1995). However, it has not been shown in infected humans that this is also the case. A clinical trial is proceeding using an IL2-diphtheria toxin (IL2-DT) chimeric construct (Seragen). Because it is unclear whether the elimination of activated T lymphocytes from patients who are already immunodeficient will not further the defect in the immune system and hasten the disease process, this clinical trial has proceeded with considerable caution. At low doses, the immunotoxin was well tolerated, with CD4 and CD8 counts unchanged during therapy (Ives et al., 1994). Although this was primarily a dose-finding and toxicity trial not designed to test efficacy, several patients in the higher dose groups had favorable responses as indicated either by increased CD4 counts or decreased viremia (Frankel et al., 1996).

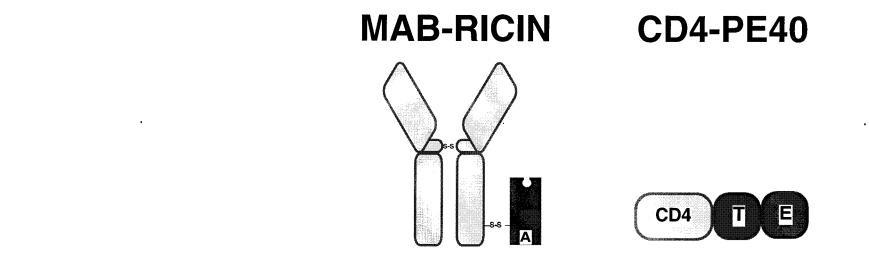
A less drastic approach is to use immunoconjugates to deliver non-cytotoxic antivirals to T cell subsets. Pokeweed antiviral protein has been chemically linked to T cell-specific antibodies. The conjugate inhibited virus production in cell cultures without causing cytotoxicity (Zarling et al., 1990). This approach has also been used with antibody-targeted liposomes containing HIV-specific antisense oligonucleotides (Renneisen et al., 1990) or reverse transcriptase inhibitors (Rombi et al., 1992). Inhibition of viral replication was seen in each case.

5. Clinical studies with CD4-PE40

For a number of reasons, CD4-PE40 appeared to be an ideal candidate for clinical testing. These include scientific rationale such as the broad reactivity of CD4-PE40 with all HIV isolates and potent in vitro activity (Chaudhary et al., 1988; Ashorn et al., 1990, 1991; Kennedy et al., 1993), as well as economic ones, such as patent coverage and production in bacterial fermentation cultures. Pharmaceutical grade CD4-PE40 was manufactured by Upjohn Laboratories and clinical trials were carried out both at the National Institutes of Health Clinical Center and by the AIDS Clinical Trials Group.

Phase I trials were performed to define dosing, toxicity, and pharmacokinetics (Davey et al., 1994; Ramachandran et al., 1994). The maximum tolerated dose was found to be 10 μg/kg, with higher doses inducing a dose-dependent elevation in hepatic aminotransferases. In contrast, ricin immunotoxins used in clinical trials in cancer have been tolerated at levels of 300–500 μg/kg (LeMaistre et al., 1991; Amlot et al., 1993; Strand et al., 1993). The serum half life of CD4-PE40 was 2–4 h. Antibodies and immunotoxins made with intact antibodies have $t_{1/2}$ values of several days, although these values are lower if there is a large tumor burden to which the administered antibody or immunotoxin adheres (Press et al., 1993). Over half of the patients developed antibodies to CD4-PE40. In the phase I trials there was no evidence of anti-HIV effect, although the trials were not designed to detect clinical efficacy. A phase II trial has been performed, although the results have not yet been published. Again, there was no evidence of any clinical effect. On the basis of these results, Upjohn Laboratories has abandoned the development of CD4-PE40.

In considering the failure of this clinical trial, several factors seem prominent. The first is the nonspecific toxicity seen at disappointingly low doses of CD4-PE40. Efficacy in treating autoimmune diseases (where the target population of lymphoid cells may be considered comparable) was only seen at 10-fold higher doses of immunotoxin. The second factor is the short $t_{1/2}$ of CD4-PE40. In trials of immunotoxins for cancer therapy, the best clinical effects were seen in patients with the greatest $t_{1/2}$. Finally, the target of CD4-PE40 is gp120, the disadvantages of which compared to gp41 have already been discussed. Fig. 2 compares the characteristics of antibody-targeted immunotoxins to CD4-PE40.



IN VITRO ACTIVITY		
CELL LINES	**++++**	**++++**
ACUTE INFECTIONS	**++**	**+++**
$T_{1/2}$	**HOURS TO DAYS**	**MINUTES TO HOURS**
MAXIMUM TOLERABLE DOSE	**>100 μg/kg**	**10 μg/kg**
ENHANCEMENT BY CD4	**+++**	**-**
CLINICAL TRIALS	**NONE**	**PHASE II**
IMMUNOGENICITY	**PROBABLY**	**YES**

Fig. 2. Comparison of the properties of CD4-PE40 and monoclonal antibody-ricin A immunotoxins. The toxic moieties are shown in darker shading. Ricin A chain is conjugated to a monoclonal antibody with a disulfide containing cross-linking reagent. The PE40 molecule consists of two domains: translocation (T) and enzymatic (E). The human anti-gp41 monoclonal antibody 2F5 would be an optimal antibody for targeting a ricin immunotoxin. This antibody recognizes a highly conserved epitope, neutralizes viral infectivity, and is not competed by patient derived antibodies. A 2F5 immunotoxin is markedly enhanced by the addition of CD4 (Pincus et al., 1996).

6. Advantages and pitfalls of immunotoxin therapy

The development of drug-resistant HIV variants has been a major factor limiting the utility of antiviral drugs in treating AIDS. Such variants can arise within weeks of the initiation of therapy. Throughout its evolution, HIV has been under continuous immunologic pressure and immunologic escape variants have been shown to occur. Despite this, there are surface-exposed epitopes on both gp120 and gp41 that have remained constant and are well-conserved on different viral isolates. Antibodies to several of these are very effective in neutralizing viral infectivity. Thus, there must be selective pressure to conserve the structure of these epitopes, presumably because mutations would result in non-infectious virions. The CD4-binding site on gp120 is one example, while the epitope recognized by the neutralizing anti-gp41 antibody 2F5 (Muster et al., 1993) is another. It is unlikely that resistant variants would arise in response to immunotoxins targeted by antibodies to these highly conserved epitopes. Our own studies with immunotoxin-resistant variants support this notion, since all HIV-variants isolated to date fail to infect other cells (Pincus et al., 1990; Duensing et al., 1995; Fang and Pincus, 1995).

As with the treatment of bacterial infections and malignancies, the introduction of combination therapy has added an important dimension to the antiviral therapy of AIDS. The use of agents with different modes of action is clearly superior to combining agents directed against the same viral system (Collier et al., 1996). The antiviral action of immunotoxins is distinctly different than that of agents currently in use or development, making immunotoxins ideal candidates for combination therapy. Synergistic activity of immunotoxins with reverse transcriptase inhibitors has been demonstrated in vitro (Ashorn et al.,

1990). Immunotoxins may also eliminate virus from lymphoid sanctuaries not eradicated by other anti-HIV agents.

Despite these advantages, there are also a number of pitfalls associated with immunotoxin therapy of AIDS. Many of these have been addressed with the extensive use of immunotoxins to treat malignancies (Frankel et al., 1996; Thrush et al., 1996). Several are unique to HIV. Table 1 summarizes the potential problems. Perhaps the most significant is the issue of immunogenicity. Even with the use of human antibodies, the toxic moieties are extremely immunogenic. However, the consequences of anti-immunotoxin antibodies are not clear. Allergic reactions are rare. Antibodies that block the action of the immunotoxin have been described, but appear to be the exception rather than the rule. Antibodies do enhance clearance of immunotoxins, but not dramatically so. A number of trials have demonstrated that effective immunotoxin therapy can proceed in the face of a strong anti-immunotoxin response. In fact, diphtheria toxins have been used in effective immunotoxins despite the fact that everyone has been immunized with diphtheria toxoid. Moreover, a number of strategies have been developed that can inhibit immunogenicity (Pincus and Tolstikov, 1995). A unique approach to eliminating immunogenicity is the use of genetic toxins, in which the DNA encoding the toxic moiety is conjugated to an antibody and expressed only in the target cell (Chen et al., 1995). If immunotoxins are shown to be effective antiviral agents, the problems associated with immunotoxin therapy will be overcome. Another form of antibodies that may adversely affect immunotoxins is patient derived antibodies directed against the same target as the immunotoxin, i.e. blocking antibodies. This may be avoided by choosing targets that are immunorecessive and do not elicit an antibody response. The 2F5 epitope of gp41 is a good example of such an epitope (Pincus et al., 1996).

Table 1
Potential problems of immunotoxin therapy in treating HIV-infection

Expense and difficulty of administration
Immunogenicity
Patient antibodies binding to the same epitope as the immunotoxin and blocking access to the target cell
Nonspecific toxicity due to toxin
Release of soluble and virion associated gp120 which may bind anti-gp120 immunotoxins and prevent them from reaching target cell
Failure of cells to express target antigens

7. Future directions

Two avenues are currently being explored. The first is the continuation of ongoing clinical trials with the chimeric IL2-DT. The other is to initiate clinical trials with an anti-HIV immunotoxin.

Phase I trials have shown that IL2-DT is safe in AIDS patients at doses that have been shown to be effective in treating autoimmune disease and malignancy. Efficacy of this immunotoxin will now be tested in Phase II trials, measuring both plasma viremia and disease progression as outcomes.

On the other hand, it is unlikely that any pharmaceutical company will pursue the development of immunotoxins targeted to HIV antigens in the absence of in vivo data indicating that the proposed immunotoxin is more effective than CD4-PE40. Because the limitations on CD4-PE40 were due to pharmacologic considerations, i.e. toxicity and pharmacokinetics, additional in vitro data (Pincus et al., 1996) will remain unconvincing. To address this in an animal model of HIV infection, we are adapting a standard model used in testing immunologic therapies for malignancy: xenografting human tumors in immunodeficient mice. The tumor we will use is a human T-cell lymphoma that is persistently infected with HIV (Pincus and Wehrly, 1990) after years in culture $>99\%$ of these cells remain productively infected with HIV. Once parameters of infection are established, the immunotoxins will be tested.

It is my belief that an anti-gp41 immunotoxin in combination with a CD4-Ig construct is most likely to be effective. Human monoclonal antibody 2F5, directed against a well-conserved, neutralizing, immunorecessive epitope is the most

likely candidate antibody for use in the immunotoxin (Muster et al., 1993; Pincus et al., 1996). An alternative CD4-based immunotoxin is also in the process of development in which an Ig molecule has been constructed with the first domain of CD4 replacing the V-domain of each H and L chain (Allaway et al., 1995). This is being conjugated to cytotoxic drugs. While this may represent an advance over CD4-PE40 by decreasing nonspecific toxicity and increasing serum half-life, the absence of CD4-mediated enhancement is still a drawback. Perhaps the ideal combination would be using this CD4-Ig immunotoxin in combination with a 2F5-based immunotoxin.

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