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*J Immunol* 2003; 170:2236-2241; doi: 10.4049/jimmunol.170.4.2236

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In Vivo Efficacy of Anti-Glycoprotein 41, But Not Anti-Glycoprotein 120, Immunotoxins in a Mouse Model of HIV Infection^1

Seth H. Pincus,^2 Hua Fang,^3 Royce A. Wilkinson,^2 Tamera K. Marcotte,^† James E. Robinson,^§ and William C. Olson^11

Immunotoxins (ITs) targeting the HIV envelope protein are among the most efficacious antiviral therapies when tested in vitro. Yet a first-generation IT targeted to gp120, CD4-PE40 (chimeric immunotoxin using CD4 and the translocation and enzymatic domains of Pseudomonas exotoxin A), showed limited promise in initial clinical testing, highlighting the need for improved ITs. We have used a new mouse model of HIV infection to test the comparative efficacy of anti-HIV ITs targeted to gp120 or to gp41. Irradiated SCID/nonobese diabetic mice are injected with a tumor of human CD4^+ cells susceptible to infection and at a separate site persistently HIV-infected cells. The spread of infection from infected to susceptible tumor is monitored by plasma p24 and the presence of HIV-infected cells in the spleen. Anti-gp41 ITs in combination with tetrameric CD4-human Ig fusion protein have pronounced anti-HIV effects. Little if any anti-HIV efficacy was found with either CD4-PE40 or an Ab-targeted anti-gp120 IT. These data support continued exploration of the utility of ITs for HIV infection, particularly the use of anti-gp41 ITs in combination with soluble CD4 derivatives. The Journal of Immunology, 2003, 170: 2236–2241.

Immunotoxins (ITs)^1 are bifunctional molecules that consist of a targeting portion and a toxic moiety. ITs have been proposed as a treatment for HIV infection. They may be targeted either to cell types known to be sites of HIV replication (1–3) or to actively infected cells expressing the HIV envelope protein(s) gp160, gp120, and gp41 on the cell surface. These anti-HIV ITs may be targeted with either CD4 (4–6) or with mAbs (7–10). In vitro studies have shown that anti-HIV ITs are among the most effective antiviral agents tested (11, 12), with efficacy against multiple cell types, including macrophages (13), and against a variety of HIV isolates (10, 14). The activity of anti-gp41 ITs, but not anti-gp120 ITs, can be enhanced 30–100-fold by the addition of soluble CD4 (sCD4) (10, 12).

Because of this promise, a clinical trial was performed with a chimeric CD4-Pseudomonas exotoxin A IT designated CD4-PE40 (chimeric immunotoxin using CD4 and the translocation and enzymatic domains of Pseudomonas exotoxin A). Because of high nonspecific toxicity and lack of clinical effect at maximum tolerated doses (MTD) (15, 16), the development of this as well as other anti-HIV ITs ceased. We have argued that CD4-PE40 is a flawed IT because of its short plasma residence time, high nonspecific toxicity, and targeting to gp120 rather than gp41 (17, 18). We have further demonstrated that a combination of an anti-gp41 IT and a CD4-based protein demonstrates superior antiviral properties in vitro (10, 12). In this manuscript we test the therapeutic potential of anti-gp120 and anti-gp41 ITs in a unique model of HIV infection, that is SCID mice injected with a mixture of HIV-infected and HIV-susceptible human CD4^+ tumor cells. The data from this model supports the further development of anti-gp41 ITs in combination with CD4-based proteins as potential treatment for HIV infection.

Materials and Methods

**Immunotoxins and CD4 derivatives**

ITs were prepared by conjugating protein G-purified mAbs to the deglycosylated ricin A chain (RAC) via the heterobifunctional cross-linking reagent SPDP (Pierce, Rockford, IL) as we have previously described (8, 10). Clinical grade deglycosylated RAC was obtained from Dr. E. Vitetta (University of Texas Southwestern Medical Center, Dallas, TX). CD4-PE40 was the gift of Upjohn Laboratories (Kalamazoo, MI). Tetrameric CD4-human Ig fusion protein (CD4-IgG2) (PRO 542), a tetrameric CD4-Ig fusion protein currently in clinical trials as an anti-HIV therapeutic (19–21), was obtained from Progenics Pharmaceuticals (Tarrytown, NY). mAb 924 is a murine IgG1 Ab directed against the V3 loop of HIV gp120 and has greatest reactivity with strains of HIV derived from the IIIB isolate (8, 9). mAbs 41.1 (9, 12) and 7B2 are human IgG1 directed against the immunodominant region of gp41 (aa 598–604).

**Cell lines**

H9, a CD4^+ human lymphoma, was obtained from Dr. M. Reitz (Institute of Human Virology, Baltimore, MD) (22). H9/NL4-3 cells are persistently infected with the NL4-3 molecular clone of HIV (23) and maintain a productive infection in virtually 100% of tissue culture cells (24). PM1 is a human CD4^+ /CCR5^− /CXCR4^+ lymphoma (25) that is permissive for infection with both R5 and X4 clinical isolates of HIV. PM1 cells persistently infected with clinical isolate (26) 208-K8 (an R5 isolate) were maintained in culture with weekly additions of uninfected PM1 cells. H1-J.C53 cells

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^1 Abbreviations used in this paper: IT, immunotoxin; CD4-IgG2, tetrameric CD4-human Ig fusion protein; CD4-PE40, chimeric immunotoxin using CD4 and the translocation and enzymatic domains of Pseudomonas exotoxin A; FIA, focal infectivity assay; IC, infectious center; MTD, maximum tolerated dose; NOD, nonobese diabetic; RAC, ricin A chain; sCD4, soluble CD4.
are HeLa cells that have been transfused with the genes that encode human CD4 and CCR5 and express both of these markers as well as CXCR4 at extremely high levels (27). These cells are permissive for infection with both R5 and X4 clinical isolates of HIV.

Focal infectivity assay (FIA)

The FIA is an extremely sensitive and quantitative assay for quantifying HIV-infected cells, termed infectious centers (ICs). The assay is capable of detecting ICs at frequencies as low as 10^{-6}. The use and experimental details of the FIA are described elsewhere (9, 10, 28). Briefly, graded numbers of cells were plated on a monolayer of H-1-J.C53 cells and washed off after 1 day. Two days later, the monolayers were fixed and permeabilized with ethanol and immunoperoxidase stained for HIV foci, using HIV-immune globulin (obtained from the AIDS Research and Reference Reagent Program, Rockville, MD) (29), followed by HRP-conjugated antimouse IgG (Zymed Laboratories, South San Francisco, CA) and the chromogenic substrate colorimetric substrate aminoethyl carbazole.

P24 Ag capture ELISA

We used an Ag capture ELISA for the measurement of plasma concentrations of the HIV core Ag, p24. The sensitivity of this assay is 5 pg/ml, and experimental details are provided elsewhere (28). Wells were coated with the HIV exotoxin A, 183-H12-SC. A 1/10 dilution of plasma treated with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) was then added and incubated overnight at 4°C. Following washing, the plates were incubated with biotin-conjugated HIV immune globulin as the detecting Ab and then with Amplex-tetraiodid HRP (Amersham Pharmacia Biotech, Piscatway, NJ). The signal was obtained with tetramethyl benzidine (Sigma-Aldrich).

Cytotoxicity assay of IT efficacy

The ability of ITs to kill HIV-infected cells in vitro was tested using a modification of our previously described cytotoxicity assay (8, 10), H9/NL-4.3 cells or uninfected H9 cells, as a control, were incubated in the presence of ITs. Viability was measured at 72 h using the MTT dye reduction assay. HIV immune globulin and macaque sera used in blocking experiments were obtained from the AIDS Research and Reference Reagent Program and S. X.-H. Hu (University of Washington, Seattle, WA), respectively.

Injection of mice with HIV-infected cells

NOD/LtSz-scid/scid mice, which carry the SCID mutation on the nonobese diabetic (NOD) background, were used at 6–12 wk of age. These SCID/NOD mice lack T and B cells and also have selective defects in NK and complement activity (30). Mice were irradiated (300 rad), and the following day they were injected with tumors in 40% Matrigel (BD Labware, Franklin Lakes, NJ). Uninfected H9 or PM1 cells (5 \times 10^6–10^7) were injected i.p.; infected H9/NL-4.3 or PM1/K8 cells (3 \times 10^5) were injected s.c. in the right flank. Mice were treated with immunotoxins by i.p. injection. At 10–12 days after injection, mice were bled and sacrificed. Plasma was used for p24 determination; spleens were removed, and the number of IC per spleen was determined using the FIA.

Measurement of plasma IT levels

The plasma concentration of ITs was measured by ELISA. A direct binding ELISA was used to detect 924-RAC. ELISA plates were coated with a synthetic peptide representing the epitope bound by Ab 924 (9). Wells were incubated with serial dilutions of test plasma, and IT binding was detected with alkaline phosphatase-conjugated antimouse IgG (Zymed Laboratories) or an alkaline phosphatase-conjugated anti-RAC mAb prepared in our laboratory and the chromogenic substrate p-nitrophenyl phosphate (Sigma-Aldrich). The IT CD4-PE40 was assayed by Ag capture ELISA with plates coated with anti-CD4 mAb Sim.2. (31) obtained from the AIDS Research and Reference Reagent Program and detected with an alkaline phosphatase-conjugated Ab to Pseudomonas exotoxin A (32). In both cases, the actual level of IT was determined by comparison to a standard curve obtained by diluting the IT in plasma.

Statistical analyses

A single-tailed alternate Welch’s t test was used to determine whether the Ab-based immunotoxins gave a statistically significant decrease in plasma p24 or number of ICs/spleen compared with either the saline or CD4-PE40 treated group. The Welch test, rather than the Student test, was chosen because the SEs of the treated groups were often significantly smaller than those of the untreated groups.

Results

Comparative efficacy, toxicity, and pharmacokinetics of two anti-gp120 ITs

Our laboratory has been involved in the development of anti-HIV ITs that are based on mAbs conjugated to RAC (9, 10, 12). Another IT, a chimera between CD4 and Pseudomonas exotoxin A, CD4-PE40, has been tested in clinical trials. It was found to be highly toxic, with no evidence of efficacy (15, 16). We have developed a mouse model to determine whether the failure was attributable to the particular IT, CD4-PE40, and whether there are circumstances in which ITs may be effective. In this model, SCID/NOD mice were injected with two tumors, one HIV infected, the other not. The spread of infection to the uninfected tumor was measured as the production of plasma p24 and the presence of HIV ICs in the spleen. In the absence of uninfected cells, there was no measurable production of p24 or ICs.

Because the major limitation of CD4-PE40 in the human trial was dose-limiting hepatotoxicity (15, 16), which was encountered at doses <10% of those tolerated for ITs in human cancer trials (33, 34), we first compared the toxicities of CD4-PE40 and mAb-RAC ITs in mice. In unirradiated mice with no tumors, the MTD of CD4-PE40 was 5 µg/mouse, with mice that received higher doses developing hepatotoxicity as evidenced by increased hepatic transaminases, decreased albumin, elevated bilirubin, asites, and focal necrosis evident upon microscopic examination of hepatic sections. In contrast, mice receiving mAb-RAC tolerated 240 µg/mouse without apparent laboratory or clinical evidence of toxicity.

In irradiated tumor-bearing mice, MTDs for both ITs were lower: CD4-PE40 was 3–3.5 µg/mouse, mAb-RAC was 35 µg/mouse. However, even at these doses, in some experiments there was toxicity that could be attributed to ITs. In particular, mortality in mice receiving 3.5 µg of CD4-PE40 three times was occasionally as high as 25%. Thus, the high level of toxicity of CD4-PE40, compared with mAb-RAC ITs, observed in humans was also seen in mice. Because hepatotoxicity was seen in uninfected mice, it is unlikely that the enhanced toxicity of CD4-PE40 in man was caused by HIV infection or an interaction between CD4-PE40 and HIV gp120.

We next compared the relative efficacy of two anti-gp120 ITs, CD4-PE40 and 924-RAC, in the murine model, so that the target molecule (i.e., gp120 vs gp41) would not be an issue in comparing the efficacy. In Fig. 1, IT efficacy was assayed as the production of viral p24 and the presence of HIV-infected cells in the spleen. CD4-PE40 was only variably effective when used at MTD, which apparently is on the cusp of an efficacious dose in this model. Because 924-RAC could be used at higher doses, it had an anti-HIV effect as measured by both plasma p24 and splenic IC values in all experiments. A combined statistical analysis of the four experiments shows that the decrease in both p24 and ICs produced by high dose 924-RAC (≥10 µg/mouse) was statistically significant when compared with saline (p < 0.0001 for p24, p = 0.007 for foci) and to CD4-PE40 (p = 0.0014 for p24, p = 0.028 for foci). CD4-PE40 did not significantly reduce either p24 (p = 0.22) or foci (p = 0.279) when compared with saline. Even at doses comparable to those used for CD4-PE40, 924-RAC was somewhat more effective than CD4-PE40 (Fig. 1C). We attribute this in part to a longer plasma residence time (see below). Thus, in this model as in humans it appears that the major barrier to successful use of CD4-PE40 is its dose-limiting toxicity.

We have proposed that another reason for the failure of CD4-PE40 is its short plasma residence time, with a t_{1/2} of ~60–90 min (15, 16), as compared with the hours to days that ITs based upon
intact Ig molecules persist in the circulation. We have also attributed the increased efficacy of 924-RAC over CD4-PE40 (Fig. 1C) to this phenomenon. To determine whether this occurs in the mice, we have measured the presence of IT in the plasma following the administration of a single 10-μg i.p. injection of either CD4-PE40 or 924-RAC (Fig. 2). The results show that plasma levels of CD4-PE40 peaked at 390 ng/ml at 1 h. By 3 and 8 h, plasma levels had decreased to 83 and 27 ng/ml, respectively, and were undetectable thereafter. In contrast, intact 924-RAC (as detected with anti-RAC mAb) peaked at 880 ng/ml at 3 h and persisted beyond 72 h. Detection of 924-RAC with anti-Ig, measuring both IT and free Ab, showed four distinct experiments. Experimental groups consisted of 8–10 mice. Error bars indicate SEM.

Because the addition of sCD4 enhances anti-gp41 ITs in vitro 30–100× without a concomitant increase in toxicity, we explored the efficacy of combined CD4 and IT therapy in vivo. We have chosen to use CD4-IgG2 because it contains an Ab Fc region, which gives it an approximately equal t1/2 (29 h in SCID mice, Ref. 35) as a mAb-based IT, and because its tetrameric nature may increase its avidity for cell-associated gp120. As shown in all four experiments in Fig. 3, the combination of CD4-IGG2 plus 41.1-RAC results in virtually complete elimination of p24 production in all experiments in doses as low as 0.5 μg/mouse. There is some breakthrough at 0.25 μg/mouse (experiment 3). This is true for both strains of HIV tested: NL4-3 (experiments 1–3) and K8 (experiment 4). Both CD4-IgG2 and 41.1-RAC show some degree of efficacy when used alone against NL4-3. It is not possible to state whether the interaction between the two agents is additive or synergistic based upon these data. Again, CD4-PE40 produces variable results. We have also examined the number of ICs per spleen in the treated animals. As with p24, combined treatment eliminates evidence of spread of infection; in experiment 1 the number of ICs per spleen was 2933 in the saline-treated group, 0 in mice receiving combined therapy, and not diminished in any of the other IT-treated groups. Similar results were obtained in other experiments: experiment 2, 12,942 vs 381 (saline vs 41.1-RAC); experiment 3, 8,020 vs 2 (in 0.5 μg/mouse group); and experiment 4, 798 vs 0. A combined statistical analysis of experiments 1–3 comparing all doses of combination CD4-IgG2 and 41.1-RAC to saline indicates highly significant inhibition of plasma p24 (p = 0.0038) and splenic ICs (p = 0.0094). The differences between saline and CD4-PE40 are not statistically significant. In experiment 4, both p24 and splenic ICs show a statistically significant inhibition when the two doses of CD4-IgG2 + 41.1-RAC are compared with saline.

We are continually screening mAbs for additional ones that may be used to target anti-HIV ITs. 7B2 is a human mAb that detects an epitope in the immunodominant region of gp41, CSGKLC (aa 598–604), which is expressed on the surface of HIV-infected cells and whose expression is increased in the presence of sCD4 (data not shown). We conjugated 7B2 to RAC and tested the activity of this IT (Fig. 4). The cytotoxic effect of 7B2-RAC on persistently infected H9/NL4-3 cells is compared with that of CD4-PE40 and 41.1-RAC in Fig. 4A. In the absence of sCD4, 7B2-RAC and CD4-PE40 have roughly equivalent cytotoxic effects, both somewhat greater than that of 41.1-RAC. In the presence of CD4-IgG2 (1 μg/ml), both anti-gp41 ITs demonstrated a marked (∼50×) enhancement of cytotoxicity. This is a true synergistic effect because the addition of CD4-IgG2 in the absence of IT causes absolutely
no cytotoxic activity (not shown). There was no cytotoxicity observed in uninfected H9 cells. The in vivo activity of 7B2-RAC combined with CD4-IgG2 is shown in Fig. 4B. Mice injected with H9/NL4-3 and H9 cells were treated with three doses of the combination therapy, saline, or the anti-gp120 IT 924-RAC. As with 41.1-RAC, the combination of 7B2-RAC with CD4-IgG2 virtually eliminated p24 production (\( p = 0.040 \) compared with saline), whereas a 15× higher dose of 924-RAC was only marginally effective. The production of splenic foci was also measured. The combination of 7B2-RAC and CD4-IgG2 lowered the mean number of IC per spleen from 492 to 81, whereas 924-RAC did not decrease ICs at all. We examined the ability of anti-HIV Abs in the serum to block the ability of the ITs to reach their targets (Fig. 4C). The data demonstrate that 7B2-RAC is somewhat less susceptible to these blocking effects than is 41.1-RAC. In summary, Abs 41.1 and 7B2 are human mAbs that bind overlapping epitopes in the immunodominant region of gp41. For the purpose of targeting ITs, 7B2 may have advantages, possibly because of increased avidity for cell-associated gp41; it produces an IT with 3× greater in vitro efficacy, and it is less susceptible to Ab-mediated blocking.

FIGURE 4. In vitro and in vivo effects of 7B2-RAC. A, The dose-response curves of the cytotoxicity of ITs on H9/NL4-3 cells, both in the presence and absence of CD4-IgG2 (1 \( \mu g/ml \)). Cytotoxicity was measured as suppression of MTT dye reduction at 72 h. B, The effect of administration of 7B2-RAC plus CD4-IgG2 in SCID/NOD mice injected with H9 and H9/NL4-3 cells. ITs were administered 1, 4, and 8 days after infection, and p24 values (mean and SEM of 14–18 animals per group) were obtained 10 d after infection. C, The effect of blocking Abs on the efficacy of 41.1-RAC and 7B2-RAC. H9/NL4-3 cells were incubated with either no serum, HIV immune globulin (6 mg/ml), or preimmune or immune sera from macaques infected with chimeric simian/human immunodeficiency virus (pool of four sera, 1/150 dilution) so that Abs in the serum that might block access of IT to its target could bind the cells. Then, either 7B2-RAC or 41.1-RAC (0.5 \( \mu g/ml \)) were added, the cells were incubated for 72 h, and MTT dye reduction was assayed.
In this paper we have used a SCID mouse model to study the in vivo efficacy of anti-HIV immunotoxins. Anti-HIV ITs are highly effective antiviral agents when tested in vitro, with a therapeutic index of >10,000, far greater than that of any other antiviral agent tested (12). There have been reports that HIV may be eliminated from tissue cultures with ITs (11). Yet when the IT CD4-PE40 was tested in humans, the clinical trial was a failure (15, 16). However, the reasons for the failure may have had more to do with the particular IT tested than with the use of anti-HIV ITs in general. We have argued that ITs can be developed with improved efficacy, tolerability, and pharmacology in vivo (17, 18). In this report we demonstrate that anti-gp41 ITs offer these advantages.

A variety of SCID mouse models have been developed to study different aspects of HIV infection. Different sources of human tissue have been used, including fetal liver and thymus, peripheral blood mononuclear cells from both HIV-infected and uninfected donors, and human T cell lymphomas. To varying degrees these models reflect physiological events that occur in human AIDS, including the depletion of thymic and CD4+ T cells (36, 37). To test the activity of antiviral drugs, the SCID mouse model has proven to be less useful than was originally hoped. This is particularly true in the models with greatest physiological relevance where it is difficult to obtain adequate numbers of reproducibly infected mice because there is either insufficient starting material (for example, fetal liver) or variable induction of infection (peripheral blood lymphocytes from different donors yield varying results in terms of engraftment and susceptibility to infection). To overcome these limitations, models have been developed that use HIV-infected tumor cells (38–43). These models, while not reflecting the pathophysiology of HIV infection, do demonstrate inhibition of HIV production by antiviral agents. Tumor cells are readily obtainable, and large numbers of mice may be used in a single experiment, thus allowing for the inclusion of multiple experimental groups and sufficient animals in each group to allow comparisons to be made. The use of tumor cell lines avoids the variability observed when different sources of primary human cells are used as the target of infection. However, as our data demonstrate, there is still experiment to experiment variability in the levels of p24 obtained, and even within experimental groups of a single experiment there are large SEs. In our model, infection must travel across body compartments from the s.c.-infected tumor to the i.p. uninfected tumor. Because the H9/NL4-3 are persistently infected, there is a continual seeding of the infection to the uninfected tumor. It is not clear whether this occurs as a result of cell-free virus or infected tumor cells.

The data from our mouse model demonstrate that a combination of CD4-IgG2 and the IT 41.1-RAC can completely eradicate evidence of HIV infection at doses as low as 0.5 μg/mouse, >70× lower than the MTD. CD4-IgG2 also showed evidence of efficacy. CD4-PE40, the IT that was used in clinical trials, was inconsistently effective at the MTD, 3.5 μg/mouse. Another gp120-targeted IT, 924-RAC, could be used at higher doses and had greater efficacy than CD4-PE40. Toxicity of CD4-PE40 was considerably higher than that of RAC containing ITs, and the type of toxicity seen was similar to that reported in the human clinical trial. The pharmacokinetics of CD4-PE40 and Ab-ricin ITs were as expected, with CD4-PE40 persisting for only a fraction of the time and reaching lower levels than the mAb-conjugated IT. These data support the notion that a combination of anti-gp41 IT plus CD4-based protein provides a superior mode of IT therapy compared with the first-generation CD4-PE40 product and other anti-gp120 ITs.

In summary, using this novel mouse model we have found that CD4-PE40 demonstrated unfavorable tolerability, poor pharmacology, and limited antiviral effects when used at its MTD. These findings parallel those seen in humans. In contrast, the combination of CD4-IgG2 and an anti-gp41 IT 41.1-RAC provided virtually complete suppression of virus infection at low doses that were well tolerated by the animals. Moreover, these immunotoxins may actually be used in doses at least 10× higher than CD4-PE40. The CD4-mediated enhancement of anti-gp41 IT activity argues that gp41 is a more appropriate target than gp120. Even when anti-gp120 ITs are used at significantly higher doses, the effect does not approach that of the combined effect of CD4 and anti-gp41 IT. Taken together these data indicate that there is still considerable potential for the use of ITs in the treatment of HIV infection. We are currently pursuing studies of IT efficacy in macaques infected with chimeric simian/human immunodeficiency virus.

Acknowledgments

We thank Shiu-Lok Hu for providing valuable advice and support for this project. We thank Lindy McLandress, Chun Liu, Corrie Cooke, Leta Eng, Tammy Jacques, and Vickie Riosas for expert technical and animal care assistance; and we thank the staff of Bozeman Deaconess Hospital Cancer Center for irradiating mice.

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