ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1993, p. 835–838
0066-4804/93/04835-04$02.00/0

Vol. 37, No. 4

Anti-Human Immunodeficiency Virus Type 1 Activity of an Anti-CD4 Immunoconjugate Containing Pokeweed Antiviral Protein

ALEJO ERICE,[1,2] HENRY H. BALFOUR, JR.,[1,3] DOROTHEA E. MYERS,[4] VALERIA L. LESKE,[1] KIM J. SANNERUD,[1] VIRGINIA KUEBELBECK,[4] JAMES D. IRVIN,[5] AND FATIH M. UCKUN[4,6]*

Departments of Laboratory Medicine and Pathology,[1] Therapeutic Radiology and Radiation Oncology,[4] Medicine,[2] Pediatrics,[3] and Pharmacology,[6] University of Minnesota Health Sciences Center, Minneapolis, Minnesota 55455, and Department of Chemistry, Southwest Texas State University, San Marcos, Texas 78666[5]

Received 21 August 1992/Accepted 8 February 1993

The ability of an αCD4-pokeweed antiviral protein (PAP) immunoconjugate to inhibit replication of human immunodeficiency virus type 1 (HIV-1) was evaluated in vitro with 22 clinical HIV-1 strains obtained from four seropositive asymptomatic individuals, three patients with AIDS-related complex, and four patients with AIDS. Fifteen isolates were from zidovudine-untreated individuals, whereas seven isolates were obtained after 24 to 104 weeks of therapy with zidovudine, alone or alternating with zalcitabine. Mean zidovudine 50% inhibitory concentrations (IC_{50}s) were 126 nM (range, 1 to 607 nM) for isolates from zidovudine-untreated individuals and 2,498 nM (range, 14 to 6,497 nM) for strains from patients treated with antiretroviral agents. Mean αCD4-PAP IC_{50}s were 48 × 10^{-3} nM (range, 0.02 × 10^{-3} to 212 × 10^{-3} nM) for isolates from zidovudine-untreated individuals, and 16 × 10^{-3} nM (range, 2 × 10^{-3} to 28 × 10^{-3} nM) for isolates from treated patients. Overall, higher concentrations of αCD4-PAP were necessary to inhibit HIV-1 strains from untreated individuals at more advanced stages of disease. Seventeen isolates were susceptible to zidovudine (mean IC_{50}, 117 nM), and five were resistant to zidovudine (mean IC_{50}, 3,724 nM). Mean αCD4-PAP IC_{50}s were 43 × 10^{-3} nM for zidovudine-susceptible isolates and 19 × 10^{-3} nM for isolates resistant to zidovudine. All HIV-1 strains had IC_{50}s greater than 0.5 nM for unconjugated PAP, the αCD19-PAP immunoconjugate, and monoclonal antibody αCD4. At concentrations as high as 5,000 nM, αCD4-PAP did not inhibit colony formation by normal bone marrow progenitor cells (BFU-E, CFU-GM, and CFU-GEMM) or myeloid cell lines (KG-1 and HL-60) and did not decrease cell viabilities of T-cell (Jurkat) or B-cell (FL-112 and Raji) precursor lines. Overall, αCD4-PAP demonstrated more potent anti-HIV-1 activity than zidovudine and inhibited replication of zidovudine-susceptible and zidovudine-resistant viruses at concentrations that were not toxic to lymphohematopoietic cell populations.

Pokeweed antiviral protein (PAP), a plant protein isolated from the leaves or seeds of *Phytolacca americana*, displays broad-spectrum antiviral activity against plant viruses, herpes simplex virus, cytomegalovirus, poliovirus, and influenza virus (1, 4, 15). In a recent study, we found that PAP conjugated to monoclonal antibodies recognizing CD4, CD5, or CD7 antigens effectively inhibited human immunodeficiency virus type 1 (HIV-1) replication in normal $CD4^+$ T cells infected with HIV-1 strain LAV_{BRU}, as well as in activated T cells from two asymptomatic HIV-1-seropositive individuals (21). These encouraging findings prompted us to evaluate the activity of an αCD4-PAP immunoconjugate further by testing its efficacy against clinical HIV-1 isolates. This report describes the in vitro activity of αCD4-PAP against clinical HIV-1 isolates, including pairs of zidovudine-susceptible and zidovudine-resistant HIV-1 strains. Susceptibilities of clinical isolates to the αCD4-PAP immunoconjugate and zidovudine were compared.

MATERIALS AND METHODS

Anti-CD4-PAP immunoconjugates. Affinity-purified monoclonal antibody αCD4 conjugated to PAP purified from spring leaves of *Phytolacca americana* was prepared as previously described (8). The purity (98%), composition, immunoreactivity, and ribosome-inhibitory activity of αCD4-PAP have been previously reported (21). Unconjugated PAP, an αCD19-PAP immunoconjugate directed against B cells, and monoclonal antibody αCD4 were used as controls.

HIV-1 isolates. HIV-1 isolates were recovered from peripheral blood leukocytes of HIV-1-infected individuals participating in National Institutes of Health-sponsored AIDS clinical trials at the University of Minnesota by using a previously described culture technique (5). Positive cultures were expanded in accordance with a standard protocol, and aliquots of stock viruses were prepared from supernatants of expanded cultures when the reverse transcriptase activity in the supernatant exceeded 20,000 cpm/50 μl (12). Stock viruses were also prepared from two pairs of zidovudine-susceptible and zidovudine-resistant HIV-1 strains (obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases; zidovudine-resistant HIV from Douglas D. Richman) (6).

Antiviral susceptibility assay. Susceptibility studies of HIV-1 isolates were performed by using a reverse transcriptase inhibition method described elsewhere (13). In brief, 5 × 10^6 phytohemagglutinin-stimulated, seronegative-

* Corresponding author.

TABLE 1. Antiviral activity of αCD4-PAP immunoconjugate and zidovudine against clinical HIV-1 strains

Group	Patient no.	HIV-1 isolate	Antiviral therapy (duration [wk])[a]	IC_{50} (nM)[b]	
				ZDV	αCD4-PAP (10^3)
Seropositive-asymptomatic	1	AT-99	None	607	3
		AT-844	None	117 (94–141)	2.5
	2	AT-87	None	26	8
		AT-1441	None	89	4
	3	AT-140	None	131	7.5
		AT-1497	None	77	24
	4	AT-345	None	1	25
		AT-1113	ZDV (48)	14	9 (11.2–6.9)
AIDS-related complex	5	AT-709	None	150	13 (7.6–20.6)
		AT-1692	None	115	46
	6	AT-332	None	135	0.02
		AT-1486	ZDV (52)	2,137	12
	7	AT-585	None	24	37
		AT-1537	ZDV (52)	1,710	10
AIDS	8	AT-1614	None	52	95
		AT-2038	ZDV-ddC (24)	84	5 (0.4–10.2)
	9	AT-1269	None	157	203
		AT-1682	ZDV-ddC (24)	4,551	2
	10	H-112-2	None	166 (64–269)	212
		G-9106	ZDV (56)	6,497 (>10,000)	42 (27.8–57)
	11	G-7623	None	44	39
		G-6912	ZDV (104)	>4,000	28 (6.3–49.2)

[a] ZDV, zidovudine; ddC, zalcitabine.
[b] Numbers in parentheses are ranges of IC_{50}s obtained in repeated susceptibility assays.

donor peripheral blood mononuclear cells were suspended in 1 ml of stock virus preparations containing 10,000 to 30,000 cpm of reverse transcriptase activity. After infection, cells were transferred to 96-well tissue culture plates (40 μl per well) containing different concentrations of αCD4-PAP (5×10^{-3} to 5 nM) or zidovudine (40 to 10,000 nM) in culture medium (RPMI 1640 supplemented with 20% fetal calf serum, 5% interleukin 2, 160 U of penicillin per ml, and 160 μg of streptomycin per ml). Each concentration was tested in triplicate. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 5 (αCD4-PAP experiments) or 10 (zidovudine experiments) days. A 5-day assay was used for the immunoconjugate experiments because higher αCD4-PAP concentrations (1.5 to 5 nM) were associated with decreased cell viabilities beginning on day 6 of exposure. At the end of the culture period, supernatants were assayed for reverse transcriptase activity. Fifty percent inhibitory concentrations (IC_{50}s) were calculated from the reverse transcriptase activities of treated versus untreated samples by using a regression analysis program (Systat; Systat Inc., Evanston, Ill.). In this assay, IC_{50}s of HIV-1 strains from patients untreated with zidovudine are less than 340 nM (17). Isolates with IC_{50}s of greater than 1,000 nM were considered highly resistant to zidovudine.

Cytotoxicity assays. Viabilities of untreated, αCD4-PAP-treated, and zidovudine-treated cells were determined in triplicate on the last day of each susceptibility assay by using a standard trypan blue dye exclusion method. In addition, the toxicity of αCD4-PAP for KG-1/HL-60 AML myeloid precursor cell lines, the FL112 fetal liver pro-B-cell line, the Raji early B-cell line, the Jurkat T-cell precursor cell line, and myeloid CFU-GM, erythroid BFU-E, and multilineage CFU-GEMM bone marrow progenitor cells was evaluated by using methylcellulose colony assays (18–20). Normal bone marrow and peripheral blood samples were procured from healthy volunteers after obtaining informed consent in accordance with the guidelines of the University of Minnesota Committee on the Use of Human Subjects in Research.

RESULTS

A total of 22 clinical HIV-1 isolates were studied, including 8 isolates from four seropositive asymptomatic individuals, 6 isolates from three patients with the AIDS-related complex, and 8 isolates from four patients with AIDS (two pairs of zidovudine-resistant and zidovudine-susceptible reference isolates were also included in this group) (Table 1). Fifteen isolates were from untreated individuals, and seven isolates were obtained after 24 to 104 weeks of treatment with zidovudine, alone or alternating with zalcitabine. The mean zidovudine IC_{50} for strains from zidovudine-untreated individuals was 126 nM (range, 1 to 607 nM), and the mean zidovudine IC_{50} for strains from zidovudine-treated patients was 2,498 nM (range, 14 to 6,497 nM). The mean αCD4-PAP IC_{50} for the 15 HIV-1 strains from zidovudine-untreated individuals was 48×10^{-3} nM (range, 0.02×10^{-3} to 212×10^{-3} nM), and the mean αCD4-PAP of the 7 HIV-1 strains from zidovudine-treated patients was 16×10^{-3} nM (range, 2×10^{-3} to 28×10^{-3} nM). All eight HIV-1 isolates from seropositive asymptomatic individuals were susceptible to zidovudine, with a mean IC_{50} of 150 nM (range, 1 to 607 nM) for isolates from zidovudine-untreated patients and an IC_{50} of 14 nM for one isolate obtained after 48 weeks of zidovudine therapy. The mean αCD4-PAP IC_{50}s in this group were 11×10^{-3} nM (range, 2.5×10^{-3} to 25×10^{-3} nM) for isolates from zidovudine-untreated individuals and 9×10^{-3} nM for the isolate obtained after 48 weeks of zidovudine therapy. In the group of HIV-1 strains from patients with the

TABLE 2. Antiviral activities of PAP, antibody αCD4, and the αCD19-PAP immunoconjugate against clinical HIV-1 strains

Group	HIV-1 isolate	IC_{50} (nM)			
		αCD4-PAP (10^3)	PAP	αCD19-PAP	αCD4
Asymptomatic	AT-99	3	>5	>5	ND[a]
AIDS-related complex	AT-709	13	>0.5	>0.5	>5
	AT-1486	12	>5	>5	ND
AIDS	AT-1682	2	>5	>5	ND
	G-9106	42	>5	>5	ND
	G-6912	28	ND	>5	ND

[a] ND, not done.

AIDS-related complex, four isolates were zidovudine susceptible (mean zidovudine IC_{50}, 106 nM; range, 24 to 150 nM), whereas two isolates obtained after 52 weeks of therapy were resistant to zidovudine (IC_{50}s, 1,710 and 2,137 nM). The mean αCD4-PAP IC_{50}s in this group were 24×10^{-3} nM (range, 0.02×10^{-3} to 46×10^{-3} nM) for isolates from untreated individuals and 11×10^{-3} nM for isolates obtained after 52 weeks of therapy. Five HIV-1 strains from patients with AIDS were zidovudine susceptible, with a mean zidovudine IC_{50} of 105 nM (range, 44 to 166 nM). Three isolates in this group obtained after 24 to 104 weeks of treatment with zidovudine, alone or alternating with zalcitabine, were highly resistant to zidovudine. The mean αCD4-PAP values in this group were 137×10^{-3} nM (range, 39×10^{-3} to 212×10^{-3} nM) for isolates from untreated individuals and 19×10^{-3} nM (range, 2×10^{-3} to 42×10^{-3} nM) for isolates from treated patients. Overall, 17 isolates were susceptible to zidovudine (mean IC_{50}, 117 nM) and 5 were resistant to zidovudine (mean IC_{50}, 3,724 nM; for one isolate, the IC_{50} could not be calculated but was >4,000 nM). The mean αCD4-PAP values were 43×10^{-3} nM for the 17 isolates susceptible to zidovudine and 19×10^{-3} nM for the 5 isolates that were resistant to zidovudine. The results of control experiments are summarized in Table 2. All of the HIV-1 isolates studied had IC_{50}s of greater than 0.5 nM for unconjugated PAP, the αCD19-PAP immunoconjugate, and monoclonal antibody αCD4.

In 18 αCD4-PAP experiments, cell viability ranged from 97.5% (for cells not exposed to the immunoconjugate) to 87.2% (for cells exposed to 5 nM αCD4-PAP). Cell viabilities in 22 zidovudine susceptibility assays ranged from 90.9% (cells not exposed to zidovudine) to 90.75% (cells exposed to 10,000 nM zidovudine). At concentrations as high as 5,000 nM, αCD4-PAP did not inhibit colony formation by normal bone marrow progenitor cells (BFU-E, CFU-GM, and CFU-GEMM) or myeloid cell lines (KG-1 and HL-60) and did not decrease the viabilities of T-cell (Jurkat) or B-cell (FL-112 and Raji) precursor lines. Mild inhibition of Jurkat cells, CFU-GEMM, and CFU-GM was seen at a 15,000 nM concentration of αCD4-PAP (29, 11, and 12%, respectively).

DISCUSSION

This study demonstrated that an αCD4-PAP immunoconjugate effectively inhibited in vitro replication of clinical HIV-1 isolates, including strains highly resistant to zidovudine. Overall, αCD4-PAP demonstrated >10,000-fold more potent anti-HIV-1 activity than zidovudine and exerted its antiviral effect at concentrations that were not toxic to lymphohematopoietic cell populations.

There was a marked heterogeneity in the susceptibilities of HIV-1 isolates to zidovudine (range of IC_{50}s, 1 to 6,497 nM), as well as to αCD4-PAP (range of IC_{50}s, 0.02×10^{-3} to 212×10^{-3} nM). Although αCD4-PAP exhibited a wide range of IC_{50}s against the clinical HIV-1 isolates studied, our results indicate that higher concentrations of the immunoconjugate were necessary to inhibit HIV-1 strains from untreated individuals who were at more advanced stages of HIV-1-related disease. We were able to evaluate seven paired HIV-1 isolates obtained before and 24 to 104 weeks after initiation of antiretroviral therapy with zidovudine or alternating zidovudine and zalcitabine. In all cases, HIV-1 strains became less susceptible to zidovudine, with isolates obtained at later time points showing 1.6- to >91-fold increases in zidovudine IC_{50}s and five isolates becoming highly resistant to zidovudine. Intriguingly, six of seven HIV-1 strains obtained from treated individuals were more susceptible to αCD4-PAP, with 1.4- to 89.0-fold lower αCD4-PAP IC_{50}s than HIV-1 isolates from the same patients obtained prior to antiretroviral therapy. Whereas αCD4-PAP effectively inhibited the replication of HIV-1, controls (including unconjugated PAP, the αCD19-PAP immunoconjugate, and monoclonal antibody αCD4) did not, indicating that the anti-HIV-1 activity of αCD4-PAP requires both the CD4 antigen-specific monoclonal antibody moiety and the antiviral PAP moiety.

Previously published studies have analyzed the anti-HIV activities of compounds designed to kill HIV-1-infected cells selectively, including anti-HIV envelope antibodies coupled to the ricin A chain (10, 11, 14) and immunoconjugates consisting of the gp120-binding region of CD4 linked to *Pseudomonas aeruginosa* exotoxin (2, 3, 16). A potential limitation of these approaches is that the heterogeneity of the viral gp120 antigen and the presence of antienvelope antibodies circulating in plasma may interfere with the binding of the conjugates to HIV-1-infected cells. In contrast, αCD4-PAP is targeted against normal antigen on $CD4^+$ cells and does not require the expression of HIV-1 envelope proteins on infected cells. Therefore, envelope heterogeneity of HIV-1 strains or the presence of plasma antienvelope antibodies should not interfere with αCD4-PAP. In addition, the reported ability of PAP to inhibit the replication of other viruses, including cytomegalovirus (4) and herpes simplex virus (1), may provide a unique advantage in the treatment of HIV-1 infections with αCD4-PAP. While extending previous studies regarding the potential of PAP immunoconjugates as a new generation of anti-HIV-1 compounds, this report supports the need to perform further in vitro studies using αCD4-PAP in combination with zidovudine (and other antiretroviral agents), as well as in vivo studies of αCD4-PAP

(alone or in combination regimens with other antiretroviral agents) using the severe combined immunodeficiency model of HIV-1 infection (7, 9).

ACKNOWLEDGMENTS

We are indebted to Marty St. Clair (Burroughs Wellcome) for helping to establish the reverse transcriptase inhibition assay, Dorothee Aeppli for statistical advice, M. J. Hildreth for editorial advice, and Tracy Bray and Christina Daugherty for secretarial assistance.

This work was supported in part by U.S. Public Health Service grant P01-CA-21737 from the National Cancer Institute, special grants from the Minnesota Medical Foundation and the Bristol-Myers Squibb Pharmaceutical Research Institute to F. M. Uckun, and U.S. Public Health Service grant P01-AI-27661 from the National Institute of Allergy and Infectious Diseases to H. H. Balfour, Jr. F. M. Uckun is a Scholar of the Leukemia Society of America.

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