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Immunology

Human immunodeficiency virus-infected T cells and monocytes are killed by monoclonal human anti-gp41 antibodies coupled to ricin A chain

(AIDS/immunotoxin)

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ABSTRACT **Two human monoclonal antibodies specific for the envelope glycoprotein (gp), gp41, of the human immunodeficiency virus were conjugated to deglycosylated ricin A chain. These immunotoxins killed human immunodeficiency virus-infected H9 (T cell) and U937 (monocyte) cell lines but were nontoxic to the uninfected cell lines or to class II-positive Daudi cells. Specific killing of infected H9 cells could be completely blocked by recombinant gp160 and partially blocked by unconjugated anti-gp41 antibody but was not blocked by recombinant gp120 or human IgG demonstrating specificity for gp41. The specific toxicity of the immunotoxins for infected U937 cells was markedly potentiated by chloroquine.**

The human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS) (1, 2). The virus infects monocytes and T cells and, as the disease progresses, other cells become involved (3–5). The major approaches to treating individuals with AIDS or HIV infection [administration of anti-viral drugs such as 3′-azido-3′-deoxythymidine (AZT) and/or immunomodulators such as interleukin 2 or interferon γ] have had limited success (for review, see ref. 4). A potential alternative therapeutic modality is the use of cytotoxic agents targeted to virus-infected cells that express HIV-encoded proteins, such as glycoprotein (gp) 120 or gp41. It has been reported that the recombinant form of the cell surface protein CD4 (which binds to a conserved site on the HIV-encoded envelope protein, gp120) coupled to ricin A chain (6) or pseudomonas exotoxin 40 (7) can kill HIV-infected lines of T cells. Another approach to targeting toxins to HIV-infected cells is to use anti-HIV antibodies. This approach is complicated by the fact that gp120 shows significant heterogeneity in structure among various isolates of HIV (8–10). Moreover, free gp120 may be shed from infected cells (11, 12) and bind to uninfected $CD4^+$ cells. This would render such cells susceptible to a targeting agent specific for gp120 (13). In contrast, gp41 is only expressed by infected cells and is highly conserved, perhaps because it is responsible for anchoring the viral protein gp120 in the plasma membrane of infected cells (8–10); thus gp41 may be a more stable target antigen. Consequently, monoclonal antibodies (mAbs) specific for gp41 are attractive targeting antibodies.

Immunotoxins (ITs) containing mAbs linked to ricin A chain (IT-As) have been utilized to specifically kill both normal and neoplastic cells *in vitro* and *in vivo* (14–16). More than 1000 patients with various malignancies or steroid-resistant graft vs. host disease have been treated with IT-As (14–17). Clinically, IT-As have shown greatest efficacy when they are targeted to lymphoid cells as, e.g., graft vs. host disease. Hence, HIV-infected cells should be susceptible to the cytotoxic effects of an appropriate IT-A. IT-As bind to target cells by way of their antibody portion after which they are endocytosed into vesicles. The A chain then translocates into the cytosol, binds to ribosomes, and catalytically inhibits protein synthesis resulting in cell death (for review, see refs. 14 and 15).

In the experiments described here, we have coupled two purified human mAbs directed against the gp41 of HIV-1 through a heterobifunctional thiol-containing cross-linker, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), to the deglycosylated A chain (dgA) of the plant toxin ricin. We have tested the cytotoxic potency of these IT-As on HIV-infected and uninfected lines of T cells (H9) and monocytes (U937).

MATERIALS AND METHODS

Preparation of Human mAbs. The two human mAbs used in these experiments are secreted by two independently derived cell lines immortalized by Epstein–Barr virus transformation of peripheral blood mononuclear cells from two HIV-seropositive patients (18). The cell lines, designated 50-69 and 98-6, secrete IgG2(κ) antibodies that are specific for the gp41 protein of HIV-1 (Fig. 1). mAbs 50-69 and 98-6 have been cloned once at 100 cells per well and twice at 10 cells per well and have been in culture since July 1987 and December 1987, respectively. Antibodies were purified from the culture supernatants by passage over Affi-Gel Blue followed by affinity chromatography on protein A-Sepharose.

Preparations of ITs Containing dgA (IT-dgA). The A chain of ricin was prepared as described (19) and was purchased from Inland Biologicals (Austin, TX). It was chemically deglycosylated since this procedure prevents an IT-A from binding to the parenchymal and nonparenchymal cells of the liver through mannose receptors (14, 15, 20).

Preparation of IT-dgAs with *N*-succinimidyl 3-(2-pyridyldithio)propionate. IT-dgAs were prepared as described (21). Briefly, *N*-succinimidyl 3-(2-pyridyldithio)propionate dissolved in dimethylformamide was added to a solution of antibody (5 mg/ml) in 0.1 M sodium phosphate buffer with 0.003 M Na_2EDTA (pH 7.5; PBE) to give a final concentra-

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Abbreviations: dgA, deglycosylated A chain; HIV, human immunodeficiency virus; IT, immunotoxin; mAb, monoclonal antibody; IT-A, IT containing mAbs linked to ricin A chain; gp (prefix), glycoprotein.

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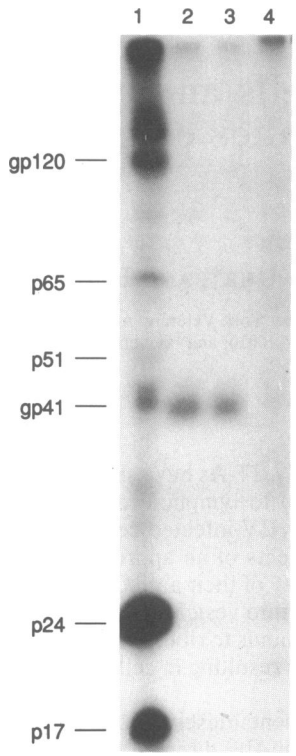

FIG. 1. Radioimmunoprecipitation of gp41 by two human mAbs. An autoradiogram of an SDS/gel is shown. Lanes: 1 and 4, bands developed using sera from HIV-seropositive and HIV-seronegative subjects, respectively; 2 and 3, gp41 immunoprecipitated by culture supernatants from cell lines 50-69 and 98-6, respectively. Positions of major viral proteins are shown on the left.

tion of 1 mM. After 30 min at room temperature, the solution was desalted on a column of Sephadex G-25 (30 × 2 cm) equilibrated with PBE. The derivatized protein was then mixed with reduced dgA chain (dissolved in PBE) using 1.3 mg of dgA chain per mg of IgG and maintained for 2 hr at 25°C and overnight at 4°C. The resulting IT-dgAs were purified on Sephacryl ACA-44 and evaluated by SDS/PAGE. The activity of the dgA component of the two anti-gp41-dgAs and the human IgG-dgA was determined by reducing the IT-dgAs with dithiothreitol and testing the ability of the released dgA to inhibit protein synthesis in a cell-free rabbit reticulocyte assay (22). Reactivity of the anti-gp41 component of each intact IT-dgA was also evaluated in a dot-blot immunoassay (J.-Y. Xu, M.K.G., and S.Z.-P., unpublished results).

Radioimmunoprecipitation. Assays were carried out according to the method of Pinter and Honnen (23). For these analyses, 30 μg of HIV (strain HTLV-IIIB) lysate (Organon Teknika–Cappel) was labeled with ^{125}I by using the Bolton–Hunter reagent (New England Nuclear). Bound and free radiolabel were separated on a Bio-Gel P-4 column (Bio-Rad) and 5 × 10^6 cpm of labeled lysate was incubated with 50 μl of undiluted human serum or culture supernatant. Fixed *Staphylococcus aureus* (Pansorbin, Calbiochem) was added to bind the immune complexes. After washing the *S. aureus* three times by centrifugation, the bacterial pellets were air-dried, resuspended in buffer, boiled for 3 min, and analyzed by electrophoresis on SDS/10% polyacrylamide gels that were dried and exposed for 1 day to Kodak X-Omat S film.

SDS/PAGE. Proteins were analyzed under both reducing and nonreducing conditions by SDS/PAGE on 10% polyacrylamide gels according to Laemmli (24). Protein bands were visualized by staining the gel with Coomassie blue. Standards for the estimation of molecular weight (Bio-Rad) were electrophoresed on the same gel.

Cytotoxicity Assays. To determine the toxicity of the two IT-dgAs, HIV (HTLV-IIIB)-infected and uninfected human cell lines, H9 (T cells) (25) and U937 (monocytes) (26) or uninfected Burkitt lymphoma (Daudi) cells were cultured with different concentrations of each experimental or control IT-dgA. Cells were used when they were in logarithmic growth. ITs were plated in triplicate in 96-well microtiter plates in complete medium containing RPMI 1640, 15% (vol/vol) fetal calf serum, and antibiotics. In some experiments, chloroquine was added to the complete medium at a final concentration of 10 μg/ml (20 μM). Cells were added at a final concentration of 3 × 10^5 cells per ml and plates were incubated for 36 hr at 37°C in 5% CO_2/95% air. Cells were pulse-labeled for 6 hr with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine[‖] and harvested on a Titertek automatic harvester (Flow Laboratories).

Inhibition of Cytotoxicity. Either 98-6-dgA or 50-69-dgA was used at a final concentration of 2.5 × 10^{-8} M. The IT-dgAs were preincubated for 1 hr at 37°C with medium, recombinant gp120, recombinant gp160, mAb 50-69, mAb 98-6, or human IgG. Cells were then added to a final concentration of 3 × 10^5/ml. Microtiter plates were incubated, pulse-labeled, and harvested as described above. Results are expressed as the percentage of [^{3}H]thymidine incorporation in untreated cells (cells with medium only).

RESULTS

Characterization of the IT-dgAs. As shown in Fig. 1, culture supernatants of both 50-69 and 98-6 immunoprecipitate gp41, but normal human serum does not. The results of SDS/PAGE analysis under nonreducing conditions of the two anti-gp41-dgAs and human IgG-dgA (control) are shown in Fig. 2. The two IT-dgAs and human IgG coupled to dgA (IgG-dgA) (control) gave major bands of the predicted molecular weights for one and two dgA chains per antibody molecule (M_r, 180,000 and 210,000, respectively) and a minor band of M_r 240,000 representing three dgA chains per antibody molecule. Free antibody was also present, but there was little or no free dgA chain. Under reducing conditions, the IT-dgAs showed characteristic bands corresponding to γ chain, light chain, and the partially deglycosylated isomers of the A chain (A_1 and A_2).

The activities of the dgA and anti-gp41 components of the two anti-gp41 dgAs and IgG-dgAs were determined after reduction with dithiothreitol. The dgAs released from all three IT-dgAs were comparable to unconjugated dgA in their ability to inhibit protein synthesis in a cell-free rabbit reticulocyte lysate assay (IC_{50} = 1–3 × 10^{-11} M). Each IT-dgA was also evaluated in a dot-blot immunoassay and both retained their anti-gp41 binding activities whereas human IgG-dgA was negative (data not shown).

Killing of HIV-Infected T Cells and Monocytes by the Anti-gp41-dgAs. To evaluate the toxicity of the two anti-gp41-dgAs, HIV-infected and uninfected human H9 cells and U937 cells were cultured with various concentrations of each experimental or control IT-dgA. As shown in Fig. 3*A*, the two specific IT-dgAs reduced protein synthesis in the infected H9 cells by 50% at a concentration of 2.0 (±0.8) × 10^{-9} M (mean ± SD in four experiments). In contrast, the control IT-dgA was not toxic to the HIV-infected H9 cells at concentrations <10^{-7} M. Furthermore, neither the specific nor nonspecific

[‖]The usual way to evaluate the toxicity of an IT-A conjugate is to pulse-label cells with [^{3}H]leucine or [^{35}S]methionine. This involves centrifuging the microtiter plates and resuspending the cells in deficient medium prior to pulse-labeling. For safety reasons, we could not do this with HIV-infected cells. Therefore, cultures were pulse-labeled with [^{3}H]thymidine.

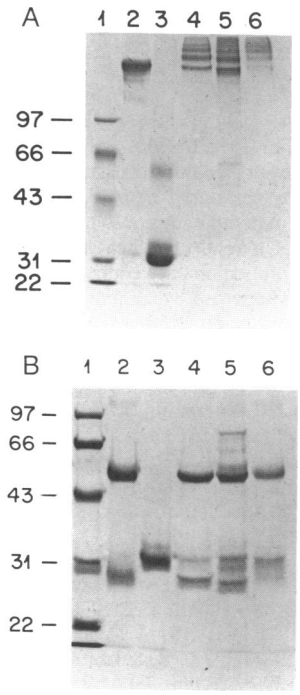

FIG. 2. SDS/PAGE of the specific and control ITs. (*A*) Unreduced gel. (*B*) Reduced gel. Lanes: 1, molecular weight markers ($\times 10^{-3}$); 2, human IgG; 3, dgA; 4, human IgG-dgA; 5, 50-69-dgA; 6, 98-6-dgA.

IT-dgAs were cytotoxic for uninfected H9 cells (Fig. 3*B*). In Fig. 3 *C* and *D*, the results of a representative experiment with U937 cells are shown. The killing of HIV-infected monocytes gave an IC_{50} value of 5 (± 2.0) $\times 10^{-9}$ M (mean $\pm$ SD in three experiments), but the absolute level of killing was variable (50–80%). To determine if the variable level of killing was due to lysosomal degradation of the dgA prior to its translocation into the cytosol, the experiments were repeated using the IT-enhancer chloroquine (27). In two experiments, the addition of chloroquine markedly potentiated the specific killing of HIV-infected U937 cells ($IC_{50} = 4–6 \times 10^{-11}$ M) and resulted in >90% killing at 10^{-8} M. Human IgG-dgA was not toxic to HIV-infected U937 cells in the presence of chloroquine and none of the conjugates was toxic to uninfected U937 with or without chloroquine.

To further demonstrate the specificity of killing, a concentration of 98-6-dgA or 50-69-dgA that would kill 74–82% of HIV-infected H9 cells (5 μg/ml) was used in the presence of recombinant gp160, recombinant gp120, or purified unconjugated 50-69, 98-6, or human IgG at 0.25–25 μg/ml. As shown in Table 1, unconjugated 50-69 and 98-6 partially blocked killing whereas recombinant gp160 was a highly effective blocker. Normal human IgG and recombinant gp120 did not block killing. Thus these data demonstrate that the anti-gp41-dgAs are cytotoxic because of their specificity for gp41.

Cytotoxicity of the Anti-gp41-dgAs for Class II$^+$ Daudi Cells. Since gp41 may have homology with class II molecules (28), we also evaluated the toxicity of the anti-gp41-dgAs on class II$^+$ Daudi cells. The IT-dgAs were not toxic to Daudi cells at concentrations as high as 10^{-7} M ($IC_{50} > 10^{-7}$ M) although an anti-class II antibody-dgA had an IC_{50} of 1.2 (± 0.2) $\times 10^{-10}$ M (mean $\pm$ SD in three experiments) (data not shown).

DISCUSSION

The results of this study demonstrate that two human monoclonal anti-gp41 antibodies (50-69 and 98-6) coupled to the deglycosylated A chain of ricin are specifically toxic to lines of HIV-infected T cells (H9) and monocytes (U937). 98-6-dgA and 50-69-dgA gave virtually identical results on HIV-infected H9 cells, killing 50% at 2×10^{-9} M and >95% of the cells at 10^{-7} M. The conjugates also killed 50% of infected monocytes at 5×10^{-9} M, but at 10^{-7} M, inhibition

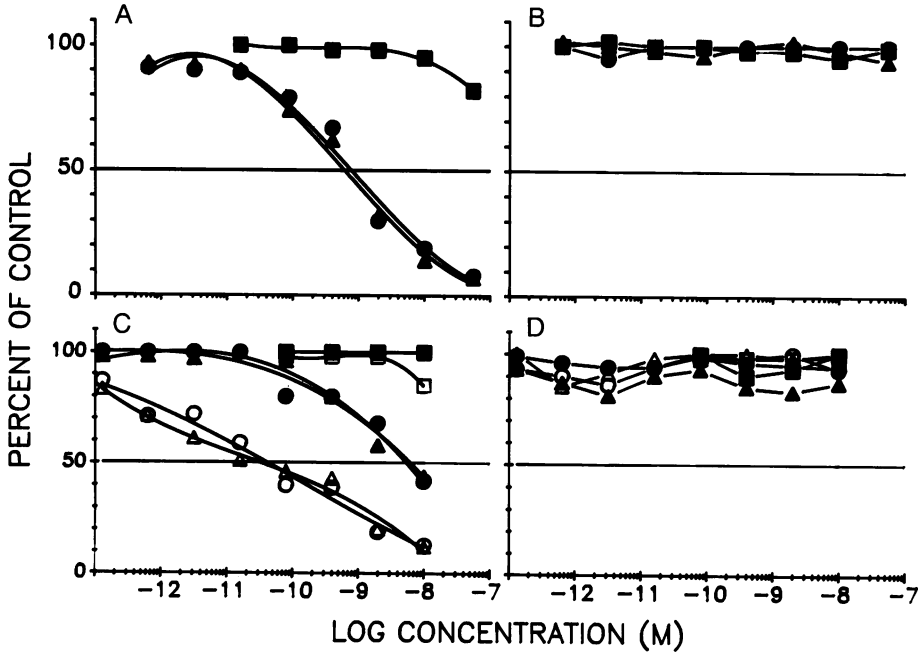

FIG. 3. Both 98-6-dgA and 50-69-dgA are toxic to HIV-infected H9 and U937 cells. ○ and ●, 98-6-dgA; △ and ▲, 50-69-dgA; □ and ■, human Ig-dgA. Cells were treated with ITs and results are expressed as a percentage of control (untreated cells). Open symbols show experiments performed in the presence of chloroquine (20 μM); closed symbols show experiments performed without chloroquine. (*A* and *B*) One representative experiment of four experiments using HIV-1-infected (*A*) and uninfected (*B*) H9 cells. (*C* and *D*) One experiment of three experiments with no chloroquine and one experiment of two experiments plus chloroquine with infected (*C*) and uninfected (*D*) U937 cells are shown. Each IT-dgA was added in serial dilution in complete medium (with or without chloroquine). Cells were then added and the assay was performed. Results are expressed as the percentage of control (untreated cells ± chloroquine).

Table 1. Cytotoxicity of 98-6-dgA and 50-69-dgA is blocked by gp160 and unconjugated anti-gp41 mAbs

Blocker	Blocker added, μg/ml	% killing			
		98-6-dgA		50-69-dgA	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
None		82	73	75	74
rgp160	25	33	0	7	0
	2.5	62	8	76	37
	0.25	79	73	78	75
rgp120	25	80	75	78	74
	2.5	82	77	76	73
	0.25	81	78	76	75
98-6	25	49	27	57	50
	2.5	78	67	75	67
	0.25	82	75	78	73
50-69	25	38	13	25	51
	2.5	70	51	70	74
	0.25	80	71	76	73
Human IgG	25	79	82	77	80
	2.5	79	77	77	77
	0.25	75	71	71	74

Either 98-6-dgA or 50-69-dAg was added at 2.5×10^{-8} M (5 μg/ml). r, recombinant. % killing = 100 − [100 × (cpm cells + IT-dgA ± blocker/cpm cells in medium only)].

varied between 50 and 80%. Chloroquine markedly potentiated the specific toxicity of the two IT-dgAs on the infected U937 cells. Chloroquine is known to potentiate the specific toxicity of IT-As (27) possibly because it increases the pH of the lysosomal compartment and inhibits the fusion of endosomes with lysosomes (29). Both effects could increase the potency of IT-As by decreasing the rate of degradation of the endocytosed IT-dgA, thereby increasing the probability that an intact dgA will translocate to the cytosol.

Of particular interest is the demonstration of (*i*) specific IT-mediated killing of HIV-infected monocytes and (*ii*) the effective use of anti-HIV-dgAs prepared with human mAbs. The killing of infected monocytes is important because there is accumulating evidence that monocytes may be the major cellular reservoir for HIV (4). Using human mAbs as part of an IT-dgA may decrease the immune response of a patient to the antibody portion of the IT-dgA. These mAbs are uncommon in their efficacy as IT-dgAs on HIV-infected cells since an indirect screening method (21) has identified only one other antibody (murine) out of more than 350 monoclonal and polyclonal antibodies tested that makes an equally potent IT-dgA (data not shown).

The reasons for the cytotoxic potency of these human anti-gp41-dgAs are not known. It is possible that binding of these IT-dgAs to a particular site(s) on the gp41 molecule induces internalization and routing of the dgA to an intracellular compartment favorable for translocation of the A chain into the cytosol. There is considerable evidence that mAbs directed against different epitopes on the same molecule as well as different molecules can vary markedly in their potency as IT-As (30, 31). In this regard, the proximity of the target epitope relative to the plasma membrane may play a role in the potency of an IT-A (31).

The extent to which these particular IT-As will effectively kill normal T cells and monocytes infected with different strains of HIV remains to be determined. However, the antibodies are group-specific rather than strain-specific since they react with gp41 molecules from two strains of HIV, HTLV-IIIB and ARV-2 (ref. 18; A. Pinter and S.Z.-P., unpublished results).

It is noteworthy that the addition of a 5-fold excess of unconjugated anti-gp41 antibody to the anti-gp41-dgAs only partially inhibited IT-induced cytotoxicity suggesting that the presence of anti-gp41 antibody in the serum of patients (32) may not be an insurmountable obstacle to therapy with ITs directed against gp41.

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