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Immunology

Anti-tumor effects of antibody–alkaline phosphatase conjugates in combination with etoposide phosphate

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ABSTRACT **Two anti-tumor monoclonal antibodies, L6 (anticarcinoma) and 1F5 (anti-B lymphoma), were covalently linked to alkaline phosphatase (AP), forming conjugates that could bind to the surface of antigen-positive tumor cells. The conjugates were capable of converting a relatively noncytotoxic prodrug, etoposide phosphate (EP), into etoposide—a drug with significant antitumor activity. *In vitro* studies with a human colon carcinoma cell line, H3347, demonstrated that while EP was less toxic than etoposide by a factor of >100, it was equally toxic when the cells were pretreated with L6–AP, a conjugate that bound to the surface of H3347 cells. The L6–AP conjugate localized in H3347 tumor xenografts in nude mice and histological evaluation indicated that the targeted enzyme (AP) was distributed throughout the tumor mass. A strong antitumor response was observed in H3347-bearing mice that were treated with L6–AP followed 18–24 hr later by EP. This response, which included the rejection of established tumors, was superior to that of EP ($P < 0.005$) or etoposide ($P < 0.001$) given alone. The 1F5–AP conjugate did not bind to H3347 cells and did not enhance the toxicity of EP on these cells *in vitro*. In addition, 1F5–AP did not localize to H3347 tumors in nude mice and did not demonstrate enhanced antitumor activity in combination with the prodrug.**

A great deal of research in recent years has been directed toward the use of tumor-associated monoclonal antibody (mAb)–drug conjugates and mAb–toxin conjugates (immunotoxins) for the treatment of cancer (1–3). This has been made possible by the availability of mAbs that recognize cell-surface antigens preferentially expressed on a variety of carcinomas, melanomas, lymphomas, and leukemias (4). Such mAbs have been used as carriers of most of the clinically used anticancer agents (5–8) and also for highly potent toxin molecules such as the A-chain toxins (3). The purpose of much of this work has been to increase the therapeutic effect of the cytotoxic agent by enhancing its localization in the target tissue and, at the same time, to spare the nontarget tissues from its toxic effects.

While some promise for this approach has been demonstrated in model systems both *in vitro* and *in vivo*, it has become apparent that there are a number of difficulties yet to be overcome. One of the most formidable problems concerning mAb targeting of clinically used anticancer drugs is that the large amount of drug required to exert a cytotoxic effect may be unobtainable because of the limitations imposed by the number of cell-surface antigens and the number of drug molecules that can be attached to each antibody. This has provided the impetus for the use of A-chain toxins, since fewer molecules are required for cytotoxic activity (3).

An additional obstacle is posed by the fact that most anticancer drugs and all A-chain toxins have special intracellular sites of activity. Immunoconjugates that are directed toward tumor-associated antigens that are not internalized (9) or that are transported to the lysosome, where the drug or toxin is degraded (3), may be of limited use. Finally, for immunoconjugates to be effective, the vast majority of the target-cell population must express the cell-surface antigen. The clonal instability and heterogeneity of tumor-cell populations complicates the use of immunoconjugates, since not all of the target cells will express the antigen, and antigen-positive cells can give rise to antigen-negative progeny (10, 11).

We wish to report here a method for the delivery of cytotoxic agents to tumor cells that has been designed to overcome the limitations imposed by low drug potency, antigen heterogeneity, and the need for antibody internalization. In this approach (Fig. 1), antibodies are used to deliver enzymes to the surface of tumor cells. The enzymes are capable of converting relatively nontoxic prodrugs, which are administered after the conjugates have bound to the cells, into active cytotoxic agents. The application of this methodology for the release of etoposide from etoposide phosphate (EP) by antibody–alkaline phosphatase (AP) conjugates is presented.

MATERIALS AND METHODS

Proteins and Cell Line. The mAbs used were L6 (IgG2a), which binds to a carbohydrate antigen on human carcinomas (12), and 1F5 (IgG2a), which is specific for the CD-20 antigen on normal and neoplastic B cells (13). AP from calf intestine was purchased from Calzyme (San Luis Obispo, CA). $NaDodSO_4$/PAGE indicated it to be a homodimer of 140 kDa. The cell line H3347 was established at Oncogen from a metastatic human colon carcinoma. L6 binds strongly to H3347 cells (saturation at 10 μg/ml), while 1F5 shows no apparent binding to these cells.

Preparation and Hydrolysis of EP. Etoposide (Bristol–Myers) was phosphorylated with an equimolar amount of phosphoryl chloride in acetonitrile and *N,N*-diisopropyl ethyl amine. The intermediate was hydrolyzed with aqueous $NaHCO_3$ and purified on a C18 silica gel column. The column was washed extensively with H_2O, and the product was then eluted with 20% (vol/vol) methanol in H_2O. The structure was confirmed through elemental analysis, NMR (^{13}C, ^{1}H, ^{31}P), and mass spectrometry.

EP (0.1 mM) in 100 mM Tris (pH 7.2) was converted quantitatively to etoposide by either free AP or antibody-bound AP (10 μg/ml) within 5 min. The reaction was monitored by HPLC using a C18 column and 50% (vol/vol) aqueous methanol as eluant. In the absence of enzyme, no hydrolysis was observed after 8 hr.

Preparation and Characterization of mAb–AP Conjugates. Conjugates were prepared by using stable thioether bonds

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Abbreviations: AP, alkaline phosphatase; EP, etoposide phosphate; mAb, monoclonal antibody.

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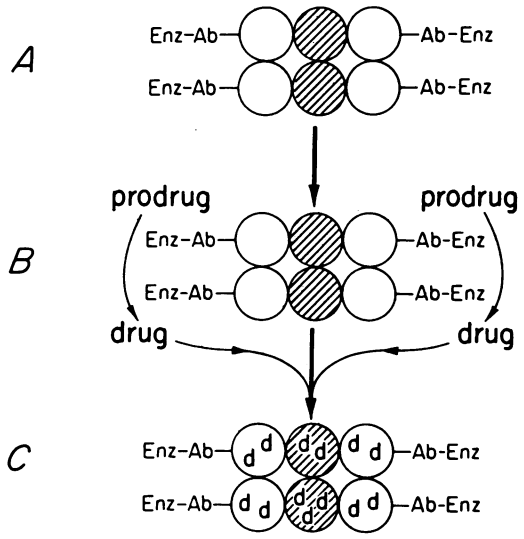

FIG. 1. Targeted enzymes for prodrug activation. (*A*) Antibody–enzyme conjugate binds to antigen positive cell population (open circles). Hatched circles, antigen-negative cells. (*B*) Enzyme converts prodrug into active drug. (*C*) Drug (d) enters cells, resulting in cell death.

according to described methods (9). Briefly, the mAbs were modified with iminothiolane (0.5 mM) to introduce a single free thiol group. AP was modified with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Pierce). The modified proteins were combined and the resulting conjugates were purified by gel filtration on S-300 Sephacryl. Conjugates purified in this manner were free of unconjugated proteins and aggregates. The concentrations were determined by absorbance at 280 nm, in which solutions (1 mg/ml) of the mAbs (160 kDa) and AP (140 kDa) absorb 1.4 and 0.76 OD units, respectively.

The enzyme activity of the conjugates was compared to unmodified AP with *p*-nitrophenyl phosphate as the substrate (14). All activity was preserved in the conjugate preparations. The antigen-binding activity on H3347 cells was measured with a Coulter Epics-C fluorescence cell analyzer and fluorescein isothiocyanate goat anti-mouse antibody as a secondary binding agent, as described (12). L6 and L6–AP bound equally well to H3347 cells, while 1F5 and 1F5–AP showed no detectable binding activity.

***In Vitro* Cytotoxicity.** A suspension of 10^6 H3347 cells in 0.1 ml of incomplete modified Dulbecco's medium (IMDM) with 10% (vol/vol) fetal calf serum was kept for 1 hr at 4°C in the presence of conjugate (5 μg/ml). The cells were washed twice with the medium containing 10% fetal calf serum, resuspended (1 ml), and plated into 96-well microtiter plates (10,000 cells per well). The drug or prodrug in IMDM was added and incubation at 37°C was commenced for 6 hr. The cells were then washed twice, and incubation was continued an additional 12 hr, followed by a 6-hr pulse with [^{3}H]thymidine (1.0 μCi per well; 1 Ci = 37 GBq). The plates were frozen at −20°C to detach the cells, and the cells were harvested onto glass fiber discs. The filters were counted on a Beckman 3701 scintillation counter.

***In Vivo* Studies.** BALB/c *nu/nu* female mice (4–6 wk old) from Life Sciences (Saint Petersburg, FL) were injected with 10^7 H3347 cells subcutaneously (s.c.) in the left and right hind flanks. The tumor cells (in IMDM) were obtained from *in vitro* cultures that had been suspended by treatment for 2 min with trypsin (0.5 g/liter) and EDTA (0.2 g/liter). They were washed twice with IMDM and incubated for 1 hr at 37°C in IMDM with 10% fetal calf serum. The cells were washed, suspended in phosphate-buffered saline (PBS), and kept at 4°C prior to injection. Both the localization and therapy studies began when the tumors reached an average size of 225 mm^3.

Localization of the Conjugates. L6 and L6–AP were labeled with ^{125}I, and 1F5 and 1F5–AP were labeled with ^{131}I by the Iodo-Gen method (15). Two days before the localization experiments, the animals were put on 0.5% (vol/vol) Lugol's iodine solution. Each mouse was injected intraperitoneally (i.p.) with 100 μg (based on each mAb) of either of the following solutions: L6–AP (5 μCi) and 1F5–AP (2.5 μCi) in 0.2 ml of PBS (pH 7.2) or a combination of L6 (5 μCi) and 1F5 (2.5 μCi) in 0.2 ml of PBS. At periodic intervals, the mice were anesthetized, bled through the orbital plexis, and sacrificed. Tissues were weighed and then assayed on a γ-counter.

Two methods were used to evaluate the level of AP activity in the tumor. Method A: The tumors from an untreated mouse, or a mouse that had been treated 24 hr earlier with L6–AP (100 μg) were washed and then gently rotated at 23°C with *p*-nitrophenyl phosphate (1 mg/ml) in 100 mM Tris (pH 9.5) containing 100 mM NaCl and 5 mM $MgCl_2$. The course of the reaction was monitored by measurement of the *p*-nitrophenol released at 410 nm, and the results were corrected for tumor weight. Method B: Excised tumors were quickly frozen to −28°C and sequential 8-μm cross-sections were made with a Reichert–Jung microtome. The phosphatase activity was measured with an AP substrate kit from Vector Laboratories (Burlingame, CA), and the results were compared to sections that were stained with hematoxylin and eosin.

***In Vivo* Tumor Therapy.** Conjugates (0.1 ml containing 300 μg of mAb in PBS), etoposide [0.2 ml containing 1.2 mg of etoposide in dimethyl sulfoxide/H_2O (2:3)], and EP (0.2 ml containing 2 mg of EP in H_2O) were administered according to the treatment schedule shown in Fig. 5. Tumor volumes were estimated by the following formula: longest length × [(perpendicular width)^2/2].

RESULTS

Preparation of the Prodrug and Conjugates. EP was prepared by condensation of phosphoryl chloride with etoposide. The product was converted to the disodium salt and was very soluble in water (Fig. 2). The susceptibility of the phosphate to enzymatic cleavage was determined by reacting 0.1 mM EP with AP (10 μg/ml), and it was found that quantitative hydrolysis occurred in <5 min.

The enzyme AP (140 kDa) was covalently linked to the mAbs L6 (12) and 1F5 (13) through a stable thioether bond. This was achieved by reacting the mAbs with iminothiolane and AP with succinimidyl 4-(*N*-maleimidomethyl)cycloxane-1-carboxylate (9). Reasonable yields (≈25%) of monomeric adducts (antibody/AP, 1:1) could be obtained by carefully controlling the degree to which the proteins were modified. The conjugates were characterized by $NaDodSO_4$/PAGE and were free of aggregated or unconjugated proteins. Furthermore, no apparent loss in enzymatic activity was observed when AP was attached to the mAb, as evidenced by the fact that the conjugates and free enzyme displayed equal activities on the substrates *p*-nitrophenyl phosphate and EP. FACS analysis served to establish that L6 and L6–AP bound equally well to the H3347 colon carcinoma cell line (saturation at mAb concentration of ≈10 μg/ml), while no detectable binding by 1F5 or 1F5–AP was observed to this cell line. The method used for conjugation thus provided well-defined material in which both the enzymatic and binding activities were preserved.

***In Vitro* Cytotoxicity.** The cytotoxic effects of etoposide and the prodrug EP were determined by measuring the incorporation of [^{3}H]thymidine into the DNA of H3347 cells. Etoposide (IC_{50}, 1 μM) was >100-fold more toxic than EP (35%

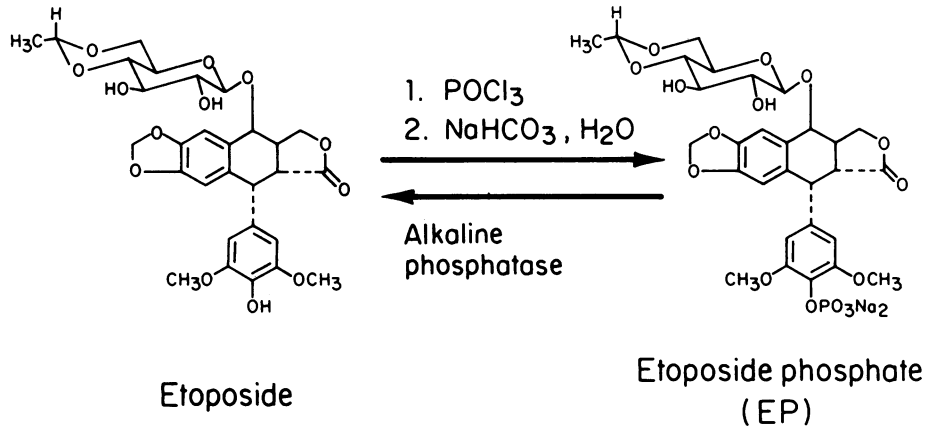

FIG. 2. Preparation of EP from etoposide and reactivity with AP.

inhibition at 100 μM; Fig. 3). Pretreatment of the cells with 1F5–AP prior to prodrug exposure resulted in no enhancement of cytotoxicity. However, a dramatic increase of cytotoxic activity was observed when the cells were first exposed to L6–AP and then to EP. The cytotoxic effect of this combination was comparable to that of etoposide alone, and it was antigen specific.

Localization of the Conjugates. *In vivo* studies were undertaken with BALB/c *nu/nu* mice that had H3347 tumors growing bilaterally. L6–AP and 1F5–AP were radiolabeled with ^{125}I and ^{131}I, respectively, by using the Iodo-Gen method (15). A comparison with ^{125}I-labeled L6 and ^{131}I-labeled 1F5 was made by injecting each mouse i.p. with either both conjugates or both mAbs and determining the ratios of specific (^{125}I) to nonspecific (^{131}I) uptake of counts in various tissues. The results for tumor and liver uptake are summarized in Table 1.

Unconjugated L6 localized efficiently to the tumor within 24 hr and remained there for at least 48 hr. During this period, the ratio of L6 to 1F5 in the tumor ranged from 8 to 12, while the ratio in the liver was quite low (1.3–1.4). The maximum level of specific uptake in the tumor for L6–AP occurred around 24 hr, at which point the ratio of L6–AP to 1F5–AP was 10.0. These results indicated that L6–AP localized within the tumor far better than did 1F5–AP but not as well as unmodified L6.

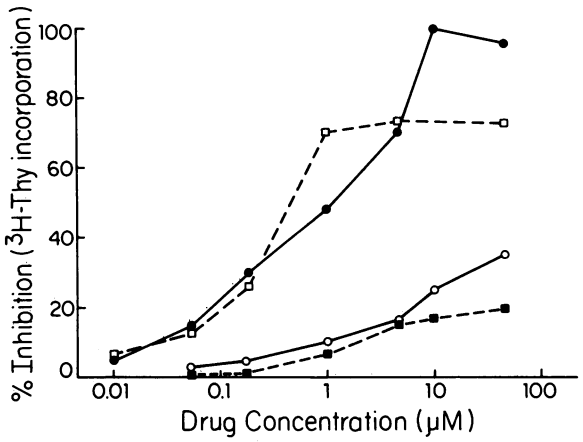

FIG. 3. Cytotoxic effects of etoposide, EP, and mAb–AP conjugates with EP as measured by inhibition of [^{3}H]thymidine incorporation into DNA. H3347 cells were exposed to etoposide or EP for 6 hr, washed, and cultured for 24 hr total (including a 6-hr pulse with [^{3}H]thymidine). Conjugate-treated cells were incubated for 1 hr with L6–AP or 1F5–AP (5 μg/ml), washed, and then exposed to EP as described above. ●, Etoposide; ○, EP; □, EP and L6–AP; ■, EP and 1F5–AP.

It was of considerable importance to determine the amount of phosphatase activity in the tumor and the degree to which this activity could be raised by targeting the enzyme with a mAb. Tumors were excised from mice that had been treated for 24 hr with L6–AP, and the total phosphatase activity was measured with *p*-nitrophenyl phosphate used as a substrate. It was found that tumors from mice that had received the L6–AP conjugate displayed as much as 10 times the level of the phosphatase activity observed in tumors from untreated mice (Fig. 4*A*).

A more detailed analysis of phosphatase activity was undertaken on cross-sections of tumors obtained from mice that had been untreated or previously treated with L6–AP or 1F5–AP. The activity was estimated by immunohistology with a phosphatase substrate that deposited a dark precipitate at the site of enzyme activity. Little activity was detected in tumors from mice that were untreated or treated with 1F5–AP (Fig. 4*B*). However, in mice that received L6–AP, phosphatase activity was greatly increased and could be seen distributed throughout the tumor. Microscopic evaluation revealed that most of the tumor cells in the L6–AP-treated mice stained highly positive for phosphatase activity.

***In Vivo* Antitumor Activity.** Therapy experiments were performed on mice that had s.c. tumors ≈225 mm^3 in volume. The conjugates L6–AP and 1F5–AP were administered (i.p.) 18–24 hr before treatment with EP. Tumor growth in these groups was compared to that in untreated mice and in mice treated with maximum tolerated doses of etoposide or EP alone. The treatment schedule and the results are shown in Fig. 5.

Etoposide had very little effect on tumor growth at the dose used, and higher doses were not well tolerated. The prodrug EP was less toxic to the animals, and the higher dose that could therefore be administered resulted in a greater antitumor effect than seen with etoposide itself. A similar degree of antitumor activity was observed in mice receiving the control conjugate 1F5–AP before treatment with EP. When, on the

Table 1. Percentage injected dose per tissue weight of administered proteins

Time after injection, hr	L6		L6–AP	
	Tumor	Liver	Tumor	Liver
2	1.6 (8.0)	4.9 (2.0)	1.5 (7.5)	5.2 (0.7)
24	3.6 (12.0)	2.3 (1.4)	1.0 (10.0)	1.3 (1.3)
48	4.0 (8.0)	2.5 (1.3)	0.5 (5.0)	0.8 (1.0)

Numbers in parentheses represent ratios of L6/1F5 or L6–AP/1F5–AP.

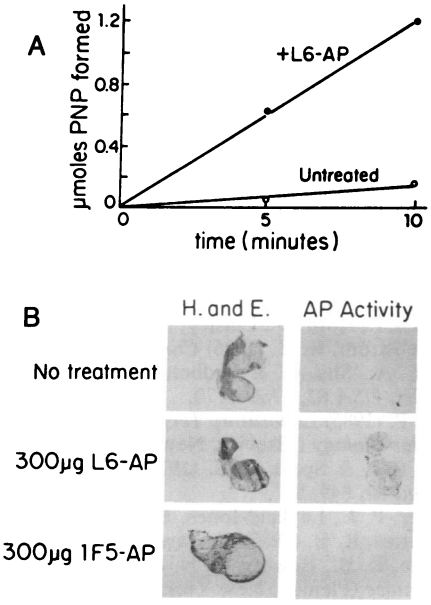

FIG. 4. Phosphatase activity in tumors that were untreated or treated with conjugates. (*A*) Non-disrupted whole tumors were suspended in a solution of *p*-nitrophenyl phosphate, and *p*-nitrophenol (PNP) release was determined at 410 nm. Treated animals received 100 μg (based on L6) of L6–AP 24 hr before tumor excision. (*B*) Tumor cross-sections (8 μm) were stained either with hemotoxylin and eosin (H. and E.) or with AP substrate (dark areas indicate high phosphatase activity). The tumors were taken from untreated mice and from mice that had been treated 24 hr earlier with 300 μg (based on mAb) of either L6–AP or 1F5–AP.

other hand, the mice were treated with L6–AP followed by EP, a much more pronounced antitumor effect was observed. L6–AP alone had no effect on tumor growth (data not shown).

A summary of the responses of each individual tumor to the therapy is shown in Table 2. Of 16 tumors in the mice treated with L6–AP and EP, 6 tumors underwent complete regression and 2 others were smaller than at the initiation of treatment. No complete or partial responses were observed in any of the other treatment protocols.

DISCUSSION

The general approach described here involves a two-step procedure in which an antibody–enzyme conjugate is first bound to noninternalizing cell-surface antigens, after which a relatively noncytotoxic prodrug is administered. The enzyme is chosen so that it can convert the prodrug into an active drug. The drug is released extracellularly where it can then diffuse into both the antigen-positive tumor cells and into nearby cells (including tumor cells) that are antigen negative. A key feature to this approach is that the bound enzyme can undergo numerous substrate turnovers, thus amplifying greatly the number of active drug molecules released in the tumor vicinity. By doing so, many of the problems associated with antigen heterogeneity and limited drug potency can be overcome.

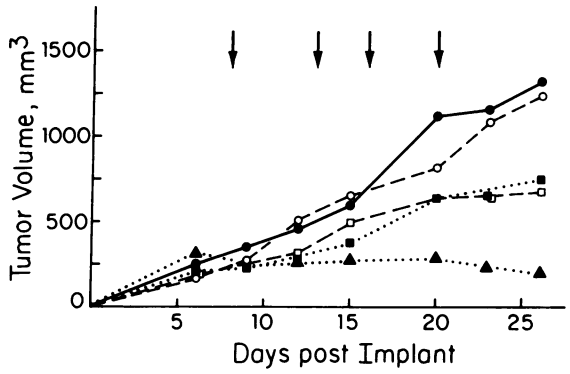

FIG. 5. Effects of etoposide, EP, and conjugates plus EP on H3347 tumors in nude mice. ●, No treatment; ○, etoposide; ■, EP; □, 1F5–AP and EP; ▲, L6–AP and EP. Arrows indicate drug treatment. Conjugates were administered 18–24 hr earlier. Each group consisted of eight mice with bilateral tumors.

Table 2. Effects of various treatments on tumor growth

Agent	Response			
	Progression	Stable	Partial	Complete
None	16	0	0	0
Etoposide	12	4	0	0
EP	6	10	0	0
1F5–AP + EP	9	7	0	0
L6–AP + EP	3	5	2	6

Data represent responses of 16 tumors in each group 23 days after tumor implant. Progression, continued tumor growth; stable, no additional tumor growth; partial, decrease in size; complete, regression leading to no apparent tumor.

The specific example involving the hydrolysis of EP by the enzyme, AP, or conjugates containing AP, demonstrates the feasibility of this strategy. Etoposide itself is cytotoxic (Fig. 3) and is used clinically for treatment of a variety of human cancers (16). The phosphate-containing prodrug EP is only weakly cytotoxic, which may be due to its inability to penetrate through the cell membrane.

Treatment of H3347 cells *in vitro* with the antigen-specific conjugate L6–AP and then with EP resulted in cytotoxic activity that was comparable to that of etoposide itself (Fig. 3). The antigen specificity of this process is indicated by the fact that EP cytotoxicity was not enhanced if the cells were pretreated with the control conjugate 1F5–AP.

Localization studies were undertaken to find out how rapidly the L6–AP conjugate accumulated in the tumor. This information was necessary to have, so that an appropriate interval between administration of the conjugate and the prodrug could be established. It was found that an appreciable level of uptake of L6–AP occurred 24 hr after conjugate treatment (Fig. 4). As expected, very little 1F5–AP localized the tumor, reflecting the fact that the conjugate does not bind to H3347 cells. Histological evaluation revealed not only that the tumor mass in animals treated with L6–AP was highly enriched in phosphatase activity, but that the conjugate had succeeded in permeating throughout the entire tumor.

The therapy involved L6–AP conjugate administration followed by prodrug (EP) treatment 18–24 hr later (Fig. 5). The results were compared to groups receiving drug, prodrug, or a nonbinding conjugate (1F5–AP) plus prodrug. A profound antitumor response was observed in animals that were treated with the combination of L6–AP and EP. This response exceeded that for the control conjugate 1F5–AP, in combination with EP, suggesting that the antigen-bound conjugate could release the active anticancer agent etoposide at the tumor site. It was surprising that EP alone had more antitumor activity than etoposide ($P < 0.05$). This might be due to the fact that the two drugs have different pharmacodistributions, and that EP releases etoposide over a period of time as it is hydrolyzed. In addition, since EP was less toxic to the mice than etoposide, it was possible to use a greater dose.

The combination of a mAb–AP conjugate and EP was chosen as a model to test the concept depicted in Fig. 1. Originally, there were some questions regarding its potential for *in vivo* use, since AP is present in many biological tissues

(17). It is apparent, however, that a significant therapeutic advantage may be gained with AP conjugates and EP. In view of the data presented here, we believe that a substantial amount of active etoposide is generated by the conjugate at the tumor site, and that consequently the tumor is exposed to a higher drug dose than could normally be achieved by systemic administration of the drug itself.

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