Locoregional Administration of Etoposide, but not of Interleukin 2, Facilitates Active Specific Immunization in Guinea Pigs with Advanced Carcinoma

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ABSTRACT

Using a guinea pig line 10 hepatocellular carcinoma model for advanced metastatic disease, we studied the therapeutic effect of local cytotoxic drug treatment at the tumor site as compared to, and in combination with, active specific immunization. In addition, locoregional treatment with interleukin 2 (IL-2) was studied. Intratumoral administration of the cytotoxic drug etoposide (VP-16), but not of IL-2, when started in a late stage of tumor growth and continued for 3 wk, caused full regression of all intradermally implanted tumors and cured a small number of animals (14%). When the primary tumor was removed at the onset of treatment, administration of VP-16 and, to a lesser degree, IL-2 at the former tumor site led to improvement of cure rates (up to 30%). Complete cure always coincided with the induction of antitumor immunity. Since both VP-16 and IL-2, when locally administered, strongly augment T-cell-mediated immune responses, the observed therapeutic effect was partially attributed to potentiation of a T-cell-mediated antitumor response. Active specific immunization (ASI) using viable irradiated tumor cells admixed with Bacillus Calmette-Guérin also aims at induction of specific antitumor immunity. In late-stage disease, ASI alone induced cure rates of 39%. Combination of ASI with local cytotoxic drug treatment, but not with locoregional administration of IL-2 at the former tumor site, led to very high cure rates (up to 78%). Cured animals were always resistant to a second challenge with line 10 tumor cells. Routinely, one systemic injection with cyclophosphamide was given at the start of all treatment protocols. Omission of CY strongly reduced the cure rates obtained with ASI and locoregional VP-16 treatment. The high cure rates likely relate to the fact that locally administered cytotoxic drugs are capable of reversing immune tolerance, besides exerting direct antitumor action within tumor-draining lymphoid tissues. The present results therefore support our view that local cytotoxic drug treatment should be further explored for its incorporation in antitumor therapies such as ASI, aiming at maximal clinical benefit and minimal toxicities.

INTRODUCTION

Death from cancer usually results from metastatic growth, since the primary tumor often can be surgically excised. Recently new support has been obtained for the view that augmentation of the host antitumor immune response can contribute to the destruction of distant (micro-)metastases. Thus, interest has grown in immunotherapeutic cancer treatment modalities. In experimental animal models, treatment with IL-2, either alone or in combination with lymphokine-activated killer or tumor-infiltrating lymphocyte infusions, frequently resulted in tumor regression and development of antitumor immunity (15). Inoculation, intratumoral administration of VP-16 or the active cyclophosphamide derivative 4-HPCY, 5 days/wk for 3 wk, cured up to 50% of the animals. Complete cure was always accompanied by the development of antitumor immunity (15). These cure rates were attributed to the potentiation of a T-cell-mediated antitumor response, in addition to the direct antitumor effect of the drug. Several findings support this view. (a) Local administration of low doses of selected cytotoxic drugs at the site of antigenic stimulation strongly potentiated the generation of effector T-cells, as detected by delayed-type hypersensitivity measurements with contact sensitizing agents in guinea pigs (16) and with proteins, RBC, or viral antigens in mice (17-19). Importantly, even a state of systemic immune tolerance could be reversed by local administration of cytotoxic drugs (20, 21). (b) In T-cell-depleted guinea pigs treated intratumorally with low doses of cytotoxic drugs, neither tumor regression nor induction of antitumor immunity occurred (Ref. 15; Footnote 4).

In clinical practice, anticancer treatment usually can only start in late-stage disease, characterized by the presence of distant metastases and a state of immunosuppression. Such immunosuppression may be mediated by both nonspecific, tumor-derived factors (22, 23) and specific immunotolerance (24, 25). Whereas surgical removal of the primary tumor mass may help to reduce nonspecific immunosuppression, local administration of cytotoxic drugs might further contribute to the reversal of specific immunotolerance and, thus, provide an effective tool for enhancing the host immune response.

The guinea pig line 10 hepatocarcinoma model permits the study of the effects of various forms of immunotherapy under...
conditions where metastases occur "naturally." In addition, tumor-induced immune suppression dominates after 14 days of tumor growth. Using this model, the purposes of the present study are to compare the therapeutic effect of local cytotoxic drug treatment with the therapeutic benefit of active specific immunotherapy in advanced disease and to study combination therapies of local drug treatment and active specific immunotherapy. In addition, the possibility of replacing cytotoxic drugs by IL-2 in local treatments is studied.

MATERIALS AND METHODS

Animals. Female albino guinea pigs of the outbred Himalayan strain were obtained from Harian Olac, Zeist, the Netherlands. Animals aged 3 to 6 mo, weighing 300 to 500 g, were used in oxazolone sensitization experiments.

Sewall-Wright inbred strain 2 guinea pigs were bred at the Department of Experimental Medicine (Free University, Amsterdam, the Netherlands). Animals of either sex, weighing 400 to 600 g, were used for tumor therapeutic studies. All animals were caged in groups of 6 and fed guinea pig ration (Hope Farms, B.V. Woerden, the Netherlands) and water ad libitum.

Tumor. The line 10 and line 1 hepatocarcinomas were originally induced in strain 2 guinea pigs by p.o. feeding with diethylnitrosamine, after which they converted to an ascites form (26). Tumor cells were collected routinely by peritoneal lavage and were used in passages 17 to 26 (line 10) and passages 94 to 97 (line 1). After i.d. inoculation into the flank (10^5 tumor cells in 0.1 ml of PBS), the line 10 tumor shows progressive growth with regional axillary lymph node metastases within 7 days. Without treatment, the animals die within 50 to 80 days from distant metastatic tumor growth.

Drugs. CY (Cyclophosphamide; Farmitalia Carlo Erba, Brussels, Belgium) and VP-16 (episepsi; Bristol Myers, SA, Zug, Switzerland) were obtained from the respective manufacturers. Human recombinant IL-2 was kindly provided by Eurocetus BV, Amsterdam, the Netherlands. Fresh frozen Tice BCG was obtained from Organon Teknika Corp., Biotechnology Research Institute, Rockville, MD.

Delayed-Type Hypersensitivity Experiments. Outbred guinea pigs were sensitized to 4-ethoxymethylene-2-phenol-oxazolone (oxazolone; BDH, Poole, United Kingdom) by epicutaneous application of 0.1 ml of 5% (w/v) oxazolone in ethanol (96%) on a shaved flanks. Injections s.c. with VP-16 or IL-2 were given daily during 7 days at the sensitization site, starting 2 days before sensitization.

Osmotic minipumps (Alzet Model 2001, kindly provided by Dr. Amkraut, ALZA, Palo Alto, CA) containing VP-16 or IL-2 were implanted 2 days before sensitization. This pump model continuously releases its content at approximately 1 /µl/h for 1 wk. The concentrations of VP-16 or IL-2 within the pumps were 4 or 20 mg/ml and 4 x 10^4 or 4 x 10^5 Cetus units/ml, respectively. Ten days after sensitization, guinea pigs were skin tested on shaved flanks, contralateral to the sensitization site, by epicutaneous application of 50 /µl of a 5% (w/v) oxazolone solution in ethanol (96%). The degree of contact sensitivity (erythema) was scored 48 h after performing the skin test by at least two independent observers, according to the following scheme: no reaction (0); pink spots (0.5); red and confluent (1.0); intense red and swollen (2.0).

Locoregional Drug Treatment. Fourteen days after i.d. tumor cell inoculation into the flank (10^4 line 10 cells), treatment was started, routinely with one single injection of CY (100 mg/kg i.p.). Intratumoral treatment with VP-16 or IL-2 was started 2 to 4 h after the CY administration. Five injections were given weekly for periods up to 3 wk (0.5 mg of VP-16/day or 10^4 Cetus units of IL-2/day). Additional locoregional treatment with VP-16 or IL-2 was given either by daily perilymphatic injections or by implantation of Alzet osmotic minipumps (Model 2002) in the tumor-draining lymph node region. This pump model continuously delivers test agents at approximately 0.5 /µl/h for a period of 2 wk. An aliquot of 0.2 ml of VP-16 or IL-2 was injected into the pump reservoir using the filling tube. The drug concentration in the pump reservoir was 20 mg/ml for VP-16 and 8 x 10^4 or 8 x 10^5 Cetus units/ml for IL-2.

In most experiments primary tumors were surgically removed at Day 14 after tumor inoculation, 2 to 4 h after CY administration. Animals were anesthetized by an i.m. injection (0.3 ml) of a mixture (1:1) of ketamine (Ketalar, 100 mg/kg; Intervet Nederland B.V., Boxmeer, the Netherlands) and xylazine (Rompun, 2%; Bayer AG, Leverkusen, Germany). In these experiments draining lymph nodes were left in situ, independent of their degree of macroscopic disease.

RESULTS

Local Administration of VP-16 or IL-2 Enhances the Development of Effector T-Cell Function in an Allergic Contact Dermatitis Model. We first verified the immunopotentiating capacity of locally administered IL-2, as compared to VP-16. Guinea pigs were sensitized to oxazolone and received IL-2 at the sensitization site either by serial injections or continuously by Alzet osmotic minipumps (Model 2001) as described in "Materials and Methods." Ten days after sensitization animals were skin tested at the opposite flank with oxazolone, and DTH reactivity was recorded by reading the skin redness (erythema). Other groups received VP-16 instead of IL-2. From the results presented in Fig. 1 it is clear that animals sensitized with oxazolone and treated with PBS injections or PBS-releasing minipumps displayed only very weak reactivity to the contact sensitizer. Whereas s.c. injections with IL-2 did not enhance this weak reactivity, s.c. administration of 10^4 or 10^5 Cetus units of IL-2 per day by continuous delivery from Alzet osmotic minipumps during 1 wk strongly enhanced the DTH reactivity to oxazolone (Fig. 1). The effect on development of skin hypersensitivity was similar to that observed with VP-16 administered in either way (Fig. 1).

Therapeutic Efficacy of Intratumoral IL-2 or VP-16 Treatment in Guinea Pigs Bearing a 14-Day Line 10 Tumor Transplant. To compare the effect of intratumoral IL-2 with intratumoral VP-16 administration on tumor regression and induction of anti-
tumor immunity, animals were inoculated with $10^4$ line 10 cells into the flank. At Day 14, treatment was routinely started with one systemic injection of CY (i.p., 100 mg/kg), 2 to 4 h before starting intratumoral treatment.

Complete regression of the primary tumor was always achieved after 3 wk of intratumoral VP-16 treatment (Fig. 2). The dose of 0.5 mg per day had been selected earlier as the optimal therapeutic dose for local VP-16 treatment (15). A small but not significant number of the animals (5 of 36; 14%) was completely cured (Table 1, Group 2). All but one of the cured animals rejected a second inoculum of tumor cells. Intratumoral treatment with IL-2 did not show any therapeutic effect. Neither regression of the primary tumor nor prevention of the outgrowth of metastases was ever achieved (Fig. 2; Table 1, Groups 3 and 4). Also no beneficial effect of perilymphatic IL-2 administration was observed when supplemented to local VP-16 treatment (Table 1, Group 5). Perilymphatic VP-16 release from minipumps for 2 wk (Model 2002), when added to serial intratumoral VP-16 injections, did not further improve cure rates but, rather, prevented development of antitumor immunity to line 10 tumor cells (Table 1, Group 6).

In conclusion, in these experiments intratumoral administration of VP-16, but not of IL-2, invariably led to the regression of the primary dermal tumors, but in most animals therapy failed because of the eventual outgrowth of distant metastases.

Therapeutic Efficacy of Perilymphatic VP-16 or IL-2 Administration after Excision of the Primary Tumor. To reduce the tumor load at the onset of treatment, in the next series of experiments we surgically removed the primary tumor and examined the therapeutic effect of perilymphatic drug treatment on metastatic disease. Perilymphatic administration of VP-16 or IL-2 was started 1 h after surgery by s.c. administration of the drugs into the former tumor site. Table 2 shows that the routinely administered CY (100 mg/kg, i.p., at Day 14), 2 to 4 h before surgery, in itself had no significant therapeutic effect (2 of 28 animals cured; Table 2, Group 1). Daily injections of VP-16 for periods of 1 to 3 wk (perilymphatically, 5 days/wk) led to significantly improved cure rates, up to 7 of 23 animals (30%; Table 2, Groups 2 and 3). Continuous perilymphatic release of VP-16 from Alzet osmotic minipumps for 2 wk (Model 2002, 0.24 mg/day) was not effective (Table 2, Group 4). It was not possible to test higher drug doses with this pump model because of the concentration of the VP-16 stock solution delivered by the manufacturer.

Perilymphatic continuous release of IL-2 showed a small, but not significant therapeutic effect (Table 2, Group 5).

In conclusion, perilymphatic treatment with VP-16 or IL-2 of (micro-)metastases remaining after excision of the primary dermal tumor prevented metastatic tumor growth in 20 to 30% of the animals, depending on dose and mode of administration. All cured animals showed resistance to rechallenge with a lethal dose of line 10 cells at Day 120 (Table 2).

Effect of Perilymphatic VP-16 or IL-2 Administration on the Therapeutic Efficacy of ASI after Excision of the Primary Tumor in Late-Stage Tumor Growth. Although perilymphatic treatment with VP-16, or with IL-2, showed some therapeutic effect in surgically treated animals, cure rates of only up to 30% could be achieved. Previously, in the same guinea pig model it had been shown that ASI cured most animals when started in early stage disease (Day 7) (7–10). We therefore decided next to compare the respective efficacies of ASI and perilymphatic VP-16 or IL-2 treatment in late-stage disease and the therapeutic benefit of combinations of both treatment modalities. Treatment was again started at Day 14 by a single systemic injection of CY, 2 to 4 h before the excision of the primary dermal tumor.

Table 3, Group 2, shows a cure rate of 39% (11 of 28) after only one vaccination with irradiated line 10 cells and BCG [107 live BCG particles + $10^4$ line 10 cells (200 Gy)] 1 day after tumor excision. This cure rate could not be improved further by adding two booster injections (first and second injections, BCG + line 10; third injection, irradiated line 10 cells only) (Table 3, Group 3). Also, delaying the first vaccination to 8 days after surgery in order to avoid possible interference of postsurgical immunosuppression with the first vaccination did not further improve the cure rate (Table 3, Group 4).

Next, it was studied whether additional perilymphatic release of IL-2 from osmotic minipumps implanted at the site draining the former (operated) tumor site further improved the ASI results. Continuous daily delivery of $10^4$ Cetus units of IL-2 by Alzet osmotic minipumps (Model 2002) did not enhance cure rates (Table 3, Groups 6 to 8). In striking contrast, 0.5 mg of VP-16, when injected daily near the former tumor site, strongly enhanced the effect of ASI, with cure rates up to 78% (Table 3, Groups 10 to 12). The same therapeutic effect was observed when VP-16 was locoregionally delivered from Alzet Model

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**Fig. 1. Effect of local administration of VP-16 or IL-2 on DTH reactivity to oxazolone. Daily administered doses are indicated. VP-16 and IL-2 were administered during sensitization at the sensitization site, either by daily injections (D) or by continuous release (for 1 wk) from osmotic minipumps (Model 2001: •). DTH reactivity (erythema) was assessed 10 days after sensitization according to the scale described in "Materials and Methods." a, $P < 0.05; b, $P < 0.005$ when compared to PBS treatment. Columns, mean; bars, SEM.**

**Table 1.**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>PBS</th>
<th>VP-16 0.1 mg</th>
<th>VP-16 0.5 mg</th>
<th>IL-2 $10^5$ U</th>
<th>IL-2 $10^6$ U</th>
</tr>
</thead>
<tbody>
<tr>
<td>erythema</td>
<td>0.00</td>
<td>0.50</td>
<td>1.00</td>
<td>0.00</td>
<td>0.50</td>
</tr>
<tr>
<td>+ sem</td>
<td></td>
<td></td>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>(48 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2.** Growth curve of the dermal, line 10 tumor in guinea pigs treated by intratumoral injections with PBS (C), VP-16 (0.5 mg/day; △), or IL-2 ($10^6$ Cetus units/day; ○). Treatment was started 14 days after tumor cell inoculation. One systemic dose of CY (100 mg/kg) preceded all local treatments. It should be noted that regression of the primary tumor was always complete following local VP-16 treatment and that the death of animals was due to the outgrowth of lymph node and distant metastases. Local IL-2 treatment did not result in regression of either primary or metastatic tumor lesions. Each group consisted of 8 animals. Points, mean; bars, SEM.
2002 minipumps (0.24 mg/day; Table 3, Group 13). Also, when ASI was started at later time points (22, 36 days), most of the animals could be cured (Table 3, Groups 11 and 12).

Thus, additional perilymphatic treatment with the cytotoxic drug VP-16, but not with IL-2, markedly added to the therapeutic effect of ASI. High cure rates were obtained and, without exception, all cured animals showed resistance to a lethal dose of viable line 10 tumor cells, but not to the antigenically distinct line 1 tumor (results not shown).

Value of the Single Injection with CY prior to Combined ASI and Perilymphatic VP-16 Treatment. In many experimental and clinical protocols, a single systemic injection with CY is included to eliminate putative suppressor cells (14, 27). On the other hand, the cytotoxic activity of the drug, when used in this dose, may add up to the elimination of tumor cells. To verify the importance of the systemic CY injection routinely given at the onset of treatment in all previous experiments, we included a group of animals treated with ASI and perilymphatic VP-16, but without the single i.p. injection of CY at Day 14. Table 4 shows that, without systemic CY treatment, ASI did not cure a single animal when started in this late stage of tumor growth. Also, the therapeutic efficacy of the combined treatment, i.e., ASI with additional locoregional VP-16 injections, was substantially reduced when the single systemic injection of CY was excluded from the treatment protocol (Table 4).

**DISCUSSION**

The present results confirm that both local chemotherapy and active specific immunization can have significant therapeutic effects in metastatic cancer, and they demonstrate that a combination of these treatment modalities provides a very powerful therapy with little toxicity. Importantly, administration of one systemic dose of CY at the onset of treatment was obligatory for achieving high cure rates. In previous studies, using the weakly immunogenic line 10 hepatocarcinoma, we demonstrated the therapeutic efficacy and the immunopotentiating effects of intratumoral cytotoxic drug administration in late-stage disease (15). When started 14 days after tumor cell inoculation and continued for 3 wk, intratumoral chemotherapy resulted in regression of the primary tumor. All cured animals showed strong delayed-type hypersensitivity reactivity and protective immunity to line 10, but not to line 1, tumor cells. Antitumor immunity was transferable to naive recipients with immune spleen cells and was T-cell dependent. Moreover, no regression of the primary tumor was observed with intratumoral chemotherapy in T-cell-depleted guinea pigs. After 3 wk of intratumoral VP-16 treatment, complete regression of the primary tumor was always achieved as well as in nontreated animals.

When therapy failed, this was because of progressive growth of lymph node and eventually distant metastases.

In the present study we first attempted to prevent metastatic escape during intratumoral VP-16 treatment by additional continuous perilymphatic delivery of VP-16 from Alzet osmotic minipumps (0.24 mg/day). In these animals the primary tumor had not been removed. Cure rates obtained with intralesional VP-16 alone were lower than described before (15), probably because of the higher in vivo passage of line 10 cells used in these experiments. Preliminary experiments had shown that i.d. inoculation of a higher passage cell line resulted in a faster metastatic growth rate (data not shown). The low cure rates obtained by local cytotoxic drug treatment alone, however, did not improve. Rather, the development of antitumor immunity was impaired (0 of 4 survivors resisted a second inoculum with line 10 cells), probably because of the exceedingly high concentration of the drug reaching the draining lymph nodes when VP-16 was simultaneously administered intralesionally and locoregionally from the pump (Table 1, Group 6). Such interference with the development of effector T-cell functions by very high doses of locally administered cytotoxic drugs had never been observed before in guinea pig immunization models. In contrast, in mouse models this was common, presumably

### Table 1 Effect of one systemic injection with CY followed by local VP-16 or IL-2 administration on tumor regression and induction of antitumor immunity

<table>
<thead>
<tr>
<th>Group</th>
<th>Intratumoral treatment*</th>
<th>Perilymphatic treatment*</th>
<th>Tumor free/ treated</th>
<th>Resistant/rechallenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td></td>
<td>0/25 (0)*</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>VP-16, 0.5 mg</td>
<td></td>
<td>5/36 (14)</td>
<td>4/5</td>
</tr>
<tr>
<td>3</td>
<td>IL-2, 10⁶ units</td>
<td></td>
<td>0/7 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>IL-2, 10⁷ units</td>
<td></td>
<td>0/6 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>VP-16, 0.5 mg</td>
<td>IL-2 pump, 10⁶ units</td>
<td>1/15 (7)</td>
<td>1/1</td>
</tr>
<tr>
<td>6</td>
<td>VP-16, 0.5 mg</td>
<td>VP-16 pump, 0.24 mg</td>
<td>4/19 (21)*</td>
<td>0/4*</td>
</tr>
</tbody>
</table>

* Treatment was started on Day 14. All animals received one single injection with CY (100 mg/kg, i.p.). Perilymphatic lymph node treatment was started at Day 14 by s.c. injections (5 days/wk, either 1 or 3 wk) or sc. implantation of Alzet 2002 minipumps (continuous release of drug for 2 wk).

**ANIMALS**

[2443]

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related to the relatively small lymphoid organs of the latter species, providing less protection to proliferating effector T-cells within the paracortical areas (17, 19, 20). Following excision of the primary tumor, perilymphatic administration of VP-16 by continuous delivery was also not therapeutically effective. However, perilymphatic administration of VP-16 by serial injections (0.5 mg/day) following excision of the primary tumor had significant antitumor effects and allowed the development of antitumor immunity. Interestingly, the effective drug levels reached in the draining lymph nodes following continuous administration from pumps were sufficient to cause strong potentiation of effector T-cell function, as demonstrated in oxazolone sensitization experiments (Fig. 1). Presumably in tumor therapy, somewhat higher doses, although not interfering with effector T-cell function, may be more effective by an additional contribution to eradication of (micro)metastases within the draining lymphoid tissues.

Studies in experimental tumor models and humans have provided data to support the view that intratumoral or perilymphatic administration of IL-2 can inhibit tumor growth and facilitate the development of antitumor immunity (28–32). In this study no significant therapeutic effects of intratumoral IL-2 injections were found. Only when IL-2 was released locally from minipumps did the therapeutic benefit (21% cures) approach that of local chemotherapy (30% cures). This parallels the results from the oxazolone sensitization experiments, in which daily local injections of IL-2 were also not effective, whereas local continuous release exerted strong adjuvant action (Fig. 1). Thus, although both IL-2 and VP-16 can act as agents that of local chemotherapy (30% cures). This parallels the results from the oxazolone sensitization experiments, in which daily local injections of IL-2 were also not effective, whereas local continuous release exerted strong adjuvant action (Fig. 1). Thus, although both IL-2 and VP-16 can act as agents for the development of T-cell-mediated immunity has been studied in depth in both guinea pig contact sensitivity and murine delayed-type hypersensitivity models. Maximum potentiation of effector T-cell functions was obtained when the drugs were administered daily for at least 2 days at the sensitization site during antigenic stimulation (16, 17, 19, 33). Several lines of evidence support the view that immunopotentiation is due to both selective elimination of suppressor cell function and augmentation of dendritic cell function. Antigen-induced lymph node hyperplasia was always further augmented by cytotoxic drug treatment. This increase was primarily due to preferential enlargement of the paracortical areas, without reduced cellular densities or viabilities (34). Augmented cell-mediated function was transferable with draining lymph node T-cells in both

### Table 3 Effect of ASI and perilymphatic treatment of the tumor draining site on metastases remaining after excision of the primary dermal line 10 tumor, 14 days after tumor cell inoculation

<table>
<thead>
<tr>
<th>Group</th>
<th>ASI days</th>
<th>Perilymphatic treatment</th>
<th>Tumor free/treated</th>
<th>Resistant/rechallenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>2/28 (7)</td>
<td>2/2</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>IL-2 pump, 10^4 units</td>
<td>11/28 (39)</td>
<td>11/11</td>
</tr>
<tr>
<td>3</td>
<td>15, 22, 29</td>
<td>IL-2 pump, 10^4 units</td>
<td>12/31 (39)</td>
<td>12/12</td>
</tr>
<tr>
<td>4</td>
<td>22, 29, 36</td>
<td>IL-2 pump, 10^4 units</td>
<td>7/26 (27)</td>
<td>7/7</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>VP-16 injection, 0.5 mg</td>
<td>6/28 (21)</td>
<td>6/6</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>VP-16 injection, 0.5 mg</td>
<td>6/20 (30)</td>
<td>6/6</td>
</tr>
<tr>
<td>7</td>
<td>15, 22, 29</td>
<td>IL-2 pump, 10^4 units</td>
<td>3/9 (33)</td>
<td>3/3</td>
</tr>
<tr>
<td>8</td>
<td>22, 29, 36</td>
<td>IL-2 pump, 10^4 units</td>
<td>5/16 (31)</td>
<td>5/5</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>VP-16 injection, 0.5 mg</td>
<td>7/23 (30)</td>
<td>7/7</td>
</tr>
<tr>
<td>10</td>
<td>15, 22, 29</td>
<td>VP-16 injection, 0.5 mg</td>
<td>18/23 (78)</td>
<td>18/18</td>
</tr>
<tr>
<td>11</td>
<td>22, 29, 36</td>
<td>VP-16 injection, 0.5 mg</td>
<td>5/9 (56)</td>
<td>5/5</td>
</tr>
<tr>
<td>12</td>
<td>36</td>
<td>VP-16 injection, 0.5 mg</td>
<td>6/8 (75)</td>
<td>6/6</td>
</tr>
<tr>
<td>13</td>
<td>15, 22, 29</td>
<td>VP-16 pump, 0.24 mg</td>
<td>7/9 (78)</td>
<td>7/7</td>
</tr>
</tbody>
</table>

* First two vaccinations with BCG and irradiated line 10 tumor cells; third vaccination irradiated line 10 cells only.
* Perilymphatic treatment was started at Day 14, 1 h after excision of the primary tumor by s.c. injections (5 days/wk for 3 wk) or by s.c. implantation of the minipump (Model 2002, continuous release of the drugs for 2 wk).
* Number of tumor free (cured) animals was recorded on Day 120.
* Animals still available were rechallenged with 10^6 viable line 10 tumor cells i.d. at the flank (contralaterally to site of primary tumor cell inoculation); animals were observed for tumor growth during 30 days.
* Numbers in parentheses, percentage.
* P < 0.005 when compared with Group 1.
* P < 0.05 when compared with Group 1.
* P < 0.005 when compared with Group 3.
* P < 0.05 when compared with Group 3.

### Table 4 Effect of single administration of CY on cure rates obtained by combined treatment of ASI and perilymphatic VP-16

<table>
<thead>
<tr>
<th>Group</th>
<th>CY</th>
<th>Perilymphatic VP-16, 0.5 mg/day</th>
<th>ASI Days</th>
<th>Tumor free/treated</th>
<th>Resistant/rechallenged</th>
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<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>18/23 (78)</td>
<td>18/18</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3/8 (37)</td>
<td>3/3</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>0/8 (0)</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* CY was given i.p. at Day 14 (100 mg/kg), 2 to 4 h before resection of the primary tumor.
* Perilymphatic treatment was started at Day 14 after resection of the primary tumor by sc. injections with VP-16 at the (former) tumor draining lymph node site (5 days/wk, 3 wks).
* Recorded on Day 120.
* Animals still available were rechallenged with 10^6 viable line 10 tumor cells i.d. at the flank (contralaterally to site of primary tumor cell inoculation); animals were observed for tumor growth during 30 days.
* P < 0.05 when compared with Group 2.
* Numbers in parentheses, percentage.
* ND, not determined.

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All animals received one injection with CY (i.p. 100 mg/kg) at Day 14. Tumor size at Day 14 was not significantly different between the groups. The mean tumor size at Day 14 was 9.5 ± 0.1 mm.
guinea pigs and mice and characterized by strongly enhanced, antigen-induced cytokine production (19). Importantly, local cytotoxic treatment reversed T (CD4\(^+\))-cell-mediated, antigen-specific suppression in mice (20, 21). Also in this guinea pig line 10 model, tumor-induced suppression of effector T-cell function was reversed by local administration of cytotoxic drugs. On the other hand, we found that lymph node-derived dendritic cells from locally cytotoxic drug-treated mice had longer dendritic protrusions and an enhanced accessory cell function in mixed-lymphocyte reaction assays (35). Since dendritic cells are pivotal in the induction of primary immune responses, facilitation of T-helper/inducer cell function by local cytotoxic drug administration may thus be partly based on stimulatory effects on dendritic cell function. Whether this holds true for the line 10 model has yet to be verified.

In early studies from Hanna and Peters (7, 8), the efficacy of active specific immunization with viable syngeneic tumor cells has been demonstrated in the guinea pig line 10 tumor model. Results from recent clinical studies in melanoma and colon carcinoma confirm that ASI can be therapeutically effective in humans although response rates usually are within the 15 to 25\% range commonly observed in immunotherapeutic studies (11–14). Notably, however, ASI does not involve severe adverse reactions and, thus, provides an attractive modality in clinical cancer treatment. We hypothesized that the development of T-cell-mediated antitumor immunity by ASI might be augmented by simultaneous immunopotentiation through local cytotoxic drug treatment at the primary tumor site. Indeed, the results obtained from the present combination therapy studies demonstrate that this combination could cure most of the animals (up to 78\%; Table 3). Interestingly, in these experiments the therapeutic effect of ASI could never be improved by locoregional IL-2. Apparently, the mechanism of immunopotentiation invoked by cytotoxic action and the direct antitumor effects within the draining lymphoid organs both provide critical contributions to tumor eradication. In these experiments, as observed in many other experimental studies, the additional single systemic administration of CY at the onset of treatment was an important preconditioning step, required for obtaining the high cure rates (Refs. 10 and 36–38; Table 4).

In conclusion, no important therapeutic effect was found when IL-2 was administered perilymphatically at the former tumor site either alone or combined with ASI. This might be due to the fact that tumor treatment was started in late-stage disease. This implies the presence of a large tumor burden and a state of immune tolerance. To our knowledge, reversal of existing specific immunotolerance has never been achieved with conventional immune adjuvants whether acting through local antigen retention (emulsions) or providing additional mitogenic signals (mycobacteria, IL-2, etc.). In contrast, administration of cytotoxic drugs such as cyclophosphamide could restore effector T-cell function in immunotolerant animals (27). Similar reversal of previously induced immunotolerance could be obtained by local cytotoxic drug administration (Refs. 20 and 21; Footnote 4). In contrast to systemic cyclophosphamide-induced immunopotentiation, which is optimal when carried out 1 to 3 days before antigen administration, local cytotoxic drug-induced immunopotentiation can be obtained by administering the drug during antigenic stimulation. This is critically important since, in malignant disease, antigen “administration” is not at one distinct point in time, in relation to which cytotoxic drug administration can be carefully scheduled. The present results therefore support our view that local cytotoxic drug treatment should be further explored for its incorporation in antitumor therapies aiming at maximal clinical benefit and minimal toxicities.

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Locoregional Administration of Etoposide, but not of Interleukin 2, Facilitates Active Specific Immunization in Guinea Pigs with Advanced Carcinoma

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