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ORIGINAL ARTICLE

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Etoposide induction of tumor immunity in Lewis lung cancer

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Abstract *Purpose*: To determine if the antineoplastic effect of etoposide includes alteration in Lewis lung cancer cells which evoke an immunologic response in C57B1/6 host mice. *Methods and results*: Of C57Bl/6 mice injected with 10^6 Lewis lung cancer (3LL) cells followed by treatment with a single 50 mg/kg dose of etoposide (VP-16), 60% survived over 60 days, in contrast to untreated control mice which died within 30 days. Approximately 40% of surviving mice rejected a subsequent challenge with 3LL. Their splenocytes protected naive mice injected with 3LL. To test if VP-16 treatment produced alterations in 3LL cells, which induce host immunity, leading to tumor rejection, C57Bl/6 mice were injected with 3LL cells that had survived an 80–90% lethal concentration of VP-16 in vitro. These cells killed 75% of recipient mice but 60% of the surviving mice rejected challenge with 3LL. Splenocytes harvested from tumor-rejecting mice protected naive mice injected with 3LL. *Conclusion*: These results support the hypothesis that in addition to its antineoplastic cytotoxic effect, VP-16 induces changes in 3LL cells which are recognized by the host immune system resulting in immune rejection of 3LL.

Keywords Etoposide · VP-16 · Tumor immunity

Introduction

One of the major limitations of cancer control is the inability of the host immune system to reject an autologous neoplasm. Chemotherapeutic approaches are often immunosuppressive and therapeutic advantage is generally based on the tumor cytotoxicity of individual drugs or combinations of drugs [13]. Our earlier work showed a link between the use of cytotoxic chemotherapy with etoposide (VP-16) and the induction of an immune response against syngeneic murine leukemia in the intact host [16]. VP-16 is an immunosuppressive topoisomerase II-inhibiting drug which induces tumor cell apoptosis and is frequently used clinically to treat a variety of tumors [1, 3, 9, 10]. We have noted that the addition of cyclosporin A to VP-16 produces CD8 T lymphocyte-mediated tumor-specific immunity in mice bearing L1210 leukemia [17]. We have extended these experiments to a spontaneously arising non-carcinogen-induced neoplasm, Lewis lung cancer (3LL), and now report that surviving mice successfully treated with VP-16, in the absence of cyclosporin A, reject challenge with 3LL. In addition, results are presented to show that VP-16 modifies 3LL cells rendering them immunogenic. These findings are submitted to support the hypothesis that VP-16-induced cytotoxic changes include cellular membrane alterations in 3LL cells which are recognized by the immune system and cause rejection of this syngeneic lung tumor.

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Materials and methods

Tumor cell line

Lewis lung carcinoma cells (3LL), kindly provided by Dr. V. Pollack (Pfizer, Groton, Ct.) were stored in liquid nitrogen. They were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 0.05 mg/ml gentamicin and 2 m*M* glutamine (FBS medium) at 37°C in an atmosphere containing 5% CO_2 and passaged by monolayer culture. The cells were harvested from exponentially growing cultures between passage 5 and 20. 3LL cells treated in vitro with VP-16 were similarly grown in the presence of 300 n*M* VP-16 (80–90% lethal dose) for 3 days prior to harvesting. In order to prepare 3LL cells free of xenogeneic serum proteins, 3LL cells cultured in FBS medium were gradually weaned from FBS in 293 SFM II medium (Life Technologies, San Diego, Calif.) and then maintained for five passages in the absence of FBS prior to their inoculation into C57B1/6 mice. Homogenized whole-cell

preparations for the detection and measurement of antibody levels against 3LL antigens by ELISA were prepared from frozen homogenized 3LL cells stored in 0.1-ml aliquots of 5×10^7 cells/ml. Aliquots of frozen 3LL cells at 1.25×10^7/ml were thawed, centrifuged for 10 min at 1500 *g* and resuspended in RPMI-1640 medium. These homogenized whole-cell preparations were stored frozen in 0.1-ml aliquots at –60°C for subsequent ELISA determinations.

In vivo experiments

Groups of ten or more C57Bl/6 mice (Jackson Laboratory, Bar Harbor, Me.) were inoculated intraperitoneally (i.p.) with 10^6 3LL cells freshly harvested from exponentially growing cultures in 0.2 ml RPMI. Each mouse was treated 24 h later with VP-16 (Vepesid, Bristol-Myers Squibb), 50 mg/kg i.p. Mice surviving for 60 days were challenged i.p. with 10^6 3LL cells.

In order to quantify the concentration of VP-16 required to progressively inhibit the proliferation of 3LL cells, we determined the ability of VP-16 to inhibit the incorporation of tritiated thymidine into 3LL cells in an in vitro 3-day assay. In vitro cytotoxicity was measured by the ability of VP-16 to inhibit tritiated thymidine incorporation into DNA. Cells were washed, counted and resuspended at 5.3×10^3 3LL cells/ml in RPMI-1640 medium supplemented with 10% FBS, 50 μg/ml gentamicin and 2 m*M* glutamine. Cell aliquots of 150 μl were placed in quadruplicate flat bottomed wells of 96-well microtiter plates. The plates were incubated overnight for cell attachment and 10 μl VP-16 at 0–350 n*M* was added, after which the plates were incubated for another 3 days. At the end of incubation, cultures were treated with 10 μl [^{3}H]thymidine for 1 h (0.15 μCi/well).

Samples were collected on glass fiber filters using an automated sample harvester and counted in an Ecoscint liquid scintillation system. VP-16 inhibitory concentrations were determined from plots of percentage inhibition of tritiated thymidine incorporation versus nanomolar VP-16 concentrations. Groups of ten or more C57 Bl/6 mice were inoculated weekly for 3 weeks with 100,000 3LL cells that had survived a 3-day exposure to 300 n*M* VP-16 in vitro. These animals were maintained untreated. Mice surviving for 60 days were challenged i.p. with 100,000 wild-type 3LL cells. Control C57Bl/6 mice were similarly inoculated with 100,000 wild-type 3LL cells. Animal care was in accordance with our Institutional Animal Care and Use Committee guidelines.

In order to determine if the spleen cells from tumor-rejecting mice could protect naive mice from 3LL, passive transfer experiments were performed. For passive transfer experiments control and long-surviving mice (over 60 days) were killed by cervical dislocation. Spleens were removed and cell suspensions prepared by mechanical disruption into Earle's balanced salt solution. Aliquots of the spleen cell suspension were counted on a hemocytometer and admixed at a ratio of 1000:1 with 100,000 3LL cells. Survival differences between control and experimental groups were subjected to Kaplan Meier survival analysis using the log-rank test.

Determination of IgG antibodies against 3LL tumor antigens by ELISA

Homogenized 3LL whole-cell suspensions were diluted in ELISA coating buffer (50 m*M* Na_2CO_3, 50 m*M* $NaHCO_3$, 3 m*M* NaN_3). Aliquots (200 μl) were added to each well of microtiter plates (Costar, provided by Fisher Scientific). The control wells to be used as blanks were coated with 200 μl ELISA coating buffer alone. The plates were covered with Saran Wrap and incubated at 37°C overnight in a tissue culture incubator. The next morning each of the coated microtiter plates was washed five times with 0.05% Tween in phosphate-buffered saline (Tween/PBS). The coated microtiter wells were blocked by adding 200 μl of a solution of 5% non-fat dried milk powder (Bordens) in Tween/PBS to each well. The plates were then incubated for 2 h at room temperature while shaking, washed five times with Tween/PBS, blotted dry on paper towels and stored in a desiccator at room temperature under a moderate vacuum for a maximum of 2 weeks. Blocking was necessary to minimize the nonspecific adsorption of proteins to the plastic microtiter plates.

Aliquots (100 ml) of dilutions of experimental or control serum in Tween/PBS were added to two columns of wells coated with dilutions of homogenized 3LL cells. One column was coated with Tween/PBS only and used as a conjugate control to test blocking and nonspecific binding. The plates were covered with Saran Wrap, incubated for 30 min at room temperature while shaking and washed five times with Tween/PBS followed by the addition of conjugate (peroxidase-labeled goat anti-mouse IgG Fc specific; Sigma, St Louis, Mo.). The plates were covered with Saran Wrap, incubated for 30 min at room temperature while shaking and washed five times and blotted dry with Tween/PBS before the addition of 100 μl/well of 3% H_2O_2 in OPD solution (57 n*M* citric acid, 97 m*M* Na_2HPO_4, 3.7 m*M* *o*-phenylenediamine). After covering the plates and incubating for an additional 30 min at room temperature, the reaction was terminated by adding 50 μl 2 *N* H_2SO_4 to each well. The optical density (OD) per well was determined with a BioRad plate reader at 492 nm.

Results

Of C57Bl/6 mice inoculated with 10^6 3LL tumor cells i.p. and 24 h later treated with 50 mg/kg of VP-16 i.p.. 60% survived beyond 60 days. On the other hand, control mice inoculated with the same dose of tumor cells but treated with saline survived a maximum of 27 days (Fig. 1A). Animals surviving more than 60 days after treatment were subsequently challenged i.p. with 10^6 3LL cells and their survival compared to that of control mice inoculated with the same number of 3LL cells (Fig. 1B). Approximately 40% of the previously VP-16-treated long-surviving mice rejected challenge with 3LL, whereas none of the control mice given 10^6 3LL cells survived beyond 30 days ($P < 0.001$).

Adoptive transfer experiments were then performed using splenocytes from long-surviving VP-16-treated mice which had rejected 3LL challenge. The mice were inoculated with 10^5 3LL cells admixed with 10^8 spleen cells. Figure 1C shows that tumor immunity could be adoptively transferred against 3LL since 80% of the mice which had received spleen cells from 3LL-rejecting mice admixed with 3LL cells reached plateau survival compared to none of the mice which had received spleen cells from control mice admixed with 3LL cells ($P < 0.001$).

In an attempt to dissect and analyze the events that occurred in 3LL-bearing mice treated with VP-16, we used 3LL cells that had survived in vitro exposure to VP-16 to evoke an immune response in intact C57Bl/6 mice. This experiment was designed to simulate the in vivo exposure of tumor cells to a concentration of VP-16 which is cytotoxic to the majority of 3LL cells but which leaves a residual cell population sublethally modified by exposure to VP-16. For this purpose, we determined the ability of VP-16 to inhibit the incorporation of tritiated thymidine into 3LL cells in an in vitro 3-day assay (Fig. 2). The morphologic features of 3LL cells exposed in vitro to 250 and 300 n*M* VP-16 (the concentration of VP-16 which left 10–20% residual viable 3LL cells) demonstrated changes consistent with apoptosis

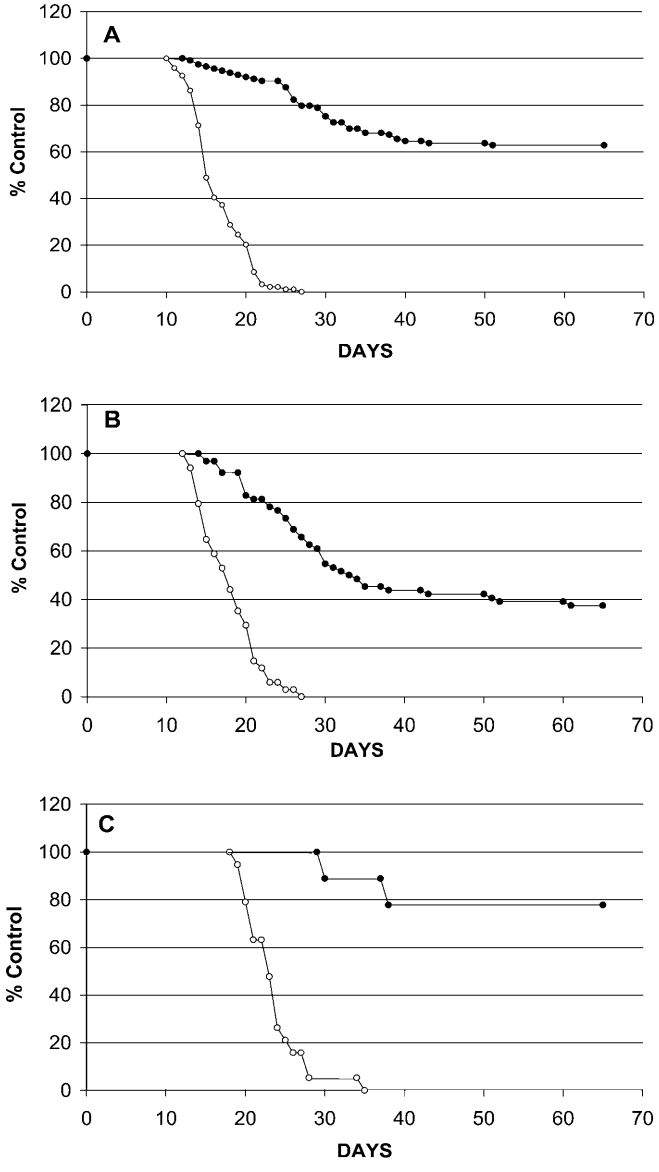

Fig. 1A–C Survival of groups of C57Bl/6 mice. **A** Initial survival of mice bearing 10^6 3LL cells following treatment with VP-16 50 mg/kg i.p. 24 h after tumor inoculation (●) compared to saline treated control mice (○) ($P < 0.001$). **B** Survival of mice challenged with 10^6 3LL surviving over 60 days (●) compared to control mice challenged with 10^6 3LL (○) ($P < 0.001$). **C** Survival of naive mice inoculated with 10^8 splenocytes harvested from 3LL-rejecting mice admixed with 10^5 3LL (●) cells compared to survival of mice inoculated with 10^8 splenocytes harvested from control mice admixed with 10^5 3LL (○) ($P < 0.001$)

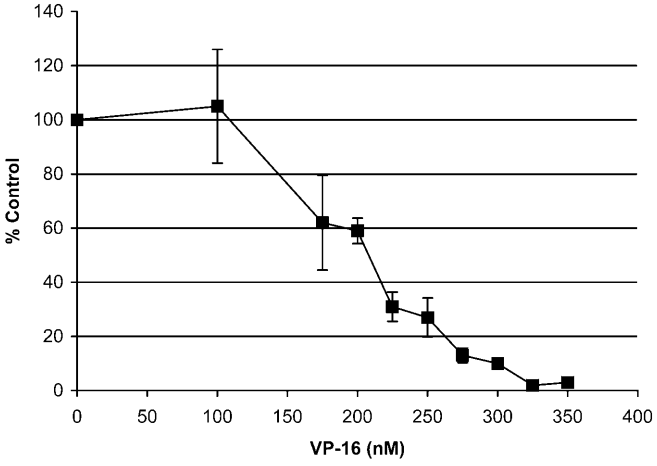

Fig. 2 VP-16 cytotoxicity assay. Dose relationship between VP-16 concentration and the inhibition of tritiated thymidine incorporation into DNA of 3LL cells

including nuclear condensation and apoptotic body development but also cellular enlargement and multinuclearity. We designated these VP-16-damaged cells as 3LL-VP (Fig. 3). Naive C57Bl/6 mice were injected with 10^5 3LL-VP cells weekly for 3 weeks. The 3LL-VP cells were prepared by incubation in 300 n*M* VP-16 for 3 days prior to each inoculation and were selected for experimental use on the basis of their ability to exclude trypan blue when visually counted. It should be noted that although these tumor inocula were washed free of VP-16 and cellular debris, they did contain 10–15% residual non-viable 3LL cells.

Figure 4 shows the pooled survival of duplicate experiments in which C57B1/6 mice were inoculated with 3LL-VP cells. Figure 4A shows that the survival of mice inoculated with 3LL-VP cells significantly exceeded that of mice inoculated once with an equivalent number (10^5) of wild-type 3LL cells ($P < 0.005$), indicating either loss of virulence of the VP-16-conditioned cells and/or a host response to them. A significant host response was apparent since 60% of surviving mice rejected challenge with 3LL ($P < 0.001$; Fig. 4B) and their splenocytes protected naive mice from 3LL in passive transfer experiments ($P < 0.001$; Fig. 4C). These results show that VP-16 promotes 3LL antigenicity.

We anticipated that the effector mechanism of tumor rejection in VP-16-treated 3LL-bearing mice would relate to cytotoxic T-lymphocyte (CTL) clones, since we had previously observed CTL-mediated tumor rejection in our earlier work with VP-16 and cyclosporin A in L1210 leukemia [17]. It was not possible, however, to demonstrate cytotoxicity of 3LL by spleen cells harvested directly from 3LL-rejecting mice or indirectly after their in vitro expansion in the presence of 3LL in a chromium-51 release assay. Since we were not able to demonstrate cellular cytotoxicity against 3LL, we examined the serum of tumor-rejecting mice for antibodies against 3LL. In order to eliminate the possible contribution of haptenic immunization with xenogeneic molecules, since 3LL cells were expanded in FBS-containing medium prior to their inoculation into host mice, we repeated experiments with 3LL cells that had been expanded in medium devoid of FBS (293 SFMII, Life Technologies). These cells behaved similarly to 3LL cells cultured in FBS medium and expanded in vivo: (1) they were uniformly fatal to untreated control mice within 30 days, (2) a single dose of VP-16 produced long survival in 60% of treated mice and (3) 40% of the long-surviving mice after VP-16 treatment rejected a

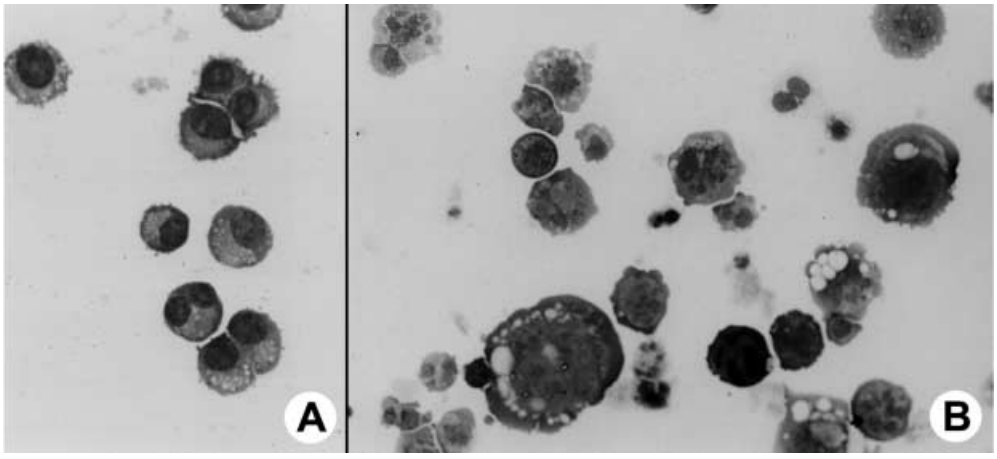

Fig. 3A, B Comparison of wild-type 3LL cells (**A**) and 3LL cells modified by exposure to 300 n*M* VP-16 for 72 h (**B**) (×400)

subsequent challenge with 10^6 3LL cells (data not shown). These observations show that the induction of tumor rejection following successful treatment of 3LL with VP-16 is independent of xenogeneic serum constituents that might be non-specifically bound to 3LL cells expanded in FBS-containing medium.

The results of two representative ELISAs comparing the reactivity of a 1:25 dilution of serum obtained from ten representative VP-16-treated mice which had survived for over 60 days following inoculation of 3LL cells expanded in serum-free medium to the reactivity of normal control C57B1/6 serum against crude membrane extracts of 3LL cells expanded in FBS-free medium are presented in Table 1. The OD of the serum from all the experimental mice tested was greater than the OD of the normal control serum. However, despite the demonstrated excess of lgG binding to 3LL crude membranes, mice numbered 2, 5, 9 and 10 failed to reject challenge with 3LL and died at 27, 39, 49 and 57 days, respectively. The remaining six mice all successfully rejected 3LL challenge.

The experimental conditions for ELISA 2 (Table 1) were the same as those for ELISA 1 in a duplicate experiment in which serum reactivity against the crude membrane extracts of syngeneic splenocytes was determined simultaneously and is also presented in Table 1. Although the reactivity of serum obtained from experimental mice against 293 SFH II-expanded 3LL crude membranes was less apparent, there was a strong correlation between the binding of experimental serum to the crude membrane extracts of 3LL cells expanded in FBS-free and freshly harvested syngeneic splenocytes

►

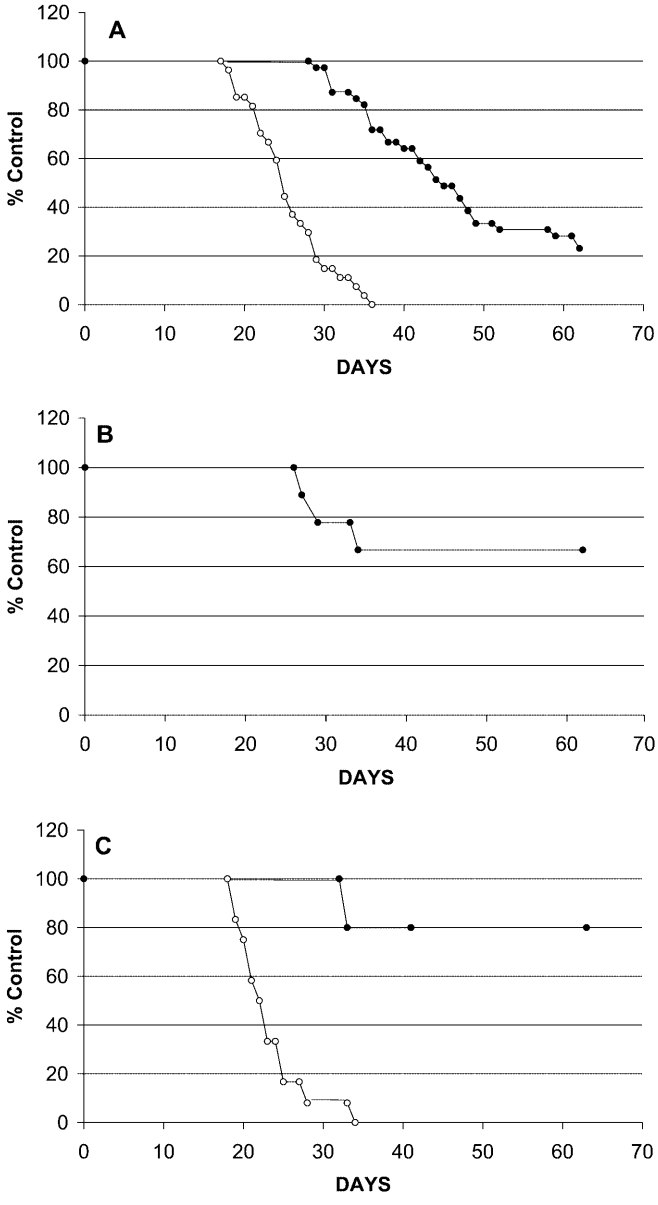

Fig. 4A–C Evidence of immunizing capacity of VP-16-conditioned 3LL cells. **A** Initial survival of mice inoculated with 10^5 VP-16-conditioned 3LL cells (●) weekly for 3 weeks compared to control mice inoculated with 10^5 wild-type 3LL cells (○) ($P < 0.005$). **B** Challenge of initial survivors (represented in **A**) with 10^5 wild-type 3LL cells (●). **C** Passive transfer of 10^8 splenocytes harvested from 3LL-rejecting mice (**B**) admixed with 10^5 wild-type 3LL cells (●) compared to passive transfer of 10^8 splenocytes harvested from control mice (○) admixed with 10^5 wild-type 3LL cells ($P < 0.001$)

Table 1 ELISA results showing binding of experimental and control mouse serum to crude membrane extracts of 3LL cells expanded in FBS-free medium and syngeneic splenocytes. There was a strong correlation between the binding of experimental serum to the extracts of 3LL cells expanded in FBS-free medium and that of freshly harvested syngeneic splenocytes ($r = 0.85$, $P < 0.002$)

Serum source	Serum reactivity (OD ± SD)		
	ELISA 1	ELISA 2	
	3LL cell membrane extract	3LL cell membrane extract	Syngeneic splenocyte membrane extract
Normal control mice	0.21 ± 0.02	0.49 ± 0.04	0.29 ± 0.02
Mice treated as in Fig. 4A			
1	0.47 ± 0.06	0.37 ± 0.01	0.27 ± 0.02
2[a]	1.25 ± 0.06	1.02 ± 0.06	3.30 ± 0.28
3	0.89 ± 0.03	0.67 ± 0.01	1.48 ± 0.02
4	0.53 ± 0.05	0.50 ± 0.02	0.33 ± 0.01
5[a]	0.62 ± 0.05	0.52 ± 0.01	0.52 ± 0.01
6	0.42 ± 0.04	0.33 ± 0.01	0.21 ± 0.01
7	0.34 ± 0.03	0.33 ± 0.01	1.91 ± 0.02
8	2.57 ± 0.18	3.37 ± 0.04	> 4
9[a]	0.46 ± 0.04	0.47 ± 0.01	0.22 ± 0.01
10[a]	1.11 ± .01	0.67 ± 0.04	1.06 ± 0.02

[a]Animals that failed to reject 3LL challenge and died

($r = 0.85$, $P < 0.002$). The reason for excessive lgG binding to both splenocyte and 3LL crude membrane extracts by serum of experimental mice compared to that by normal control serum is unclear. This observation may reflect non-specific hypergammaglobulinemia of tumor-bearing VP-16-treated mice. These findings, however, argue strongly against a role of tumor-specific antibodies in rejection of 3LL by mice successfully treated with VP-16.

Discussion

Our experiments show that a significant number of C57Bl/6 mice successfully treated for 3LL with VP-16 rejected challenge with 3LL. Their splenocytes protected naive mice injected with 3LL. To test the hypothesis that the cellular alterations caused by VP-16 treatment as 3LL cells lose viability in vivo are recognized by the host immune system leading to the rejection of 3LL, C57Bl/6 mice were inoculated with 3LL cells that had survived an 80–90% lethal concentration of VP-16 in vitro. These cells were fatal to 75% of recipient mice. However, 60% of the surviving mice rejected challenge with wild-type 3LL. Furthermore, their spleen cells protected naive mice against 3LL. This indicates that 3LL exposure to VP-16 in vitro causes changes which activate immune recognition in vivo and renders a significant proportion of mice resistant to 3LL challenge. In these experiments the inocula of 3LL cells was quantitated in terms of their ability to exclude trypan blue after in vitro exposure to 300 n*M* VP-16. Although the tumor inocula were washed free of VP-16 and cellular debris, they did contain 10–15% non-trypan blue-excluding 3LL cells. The actual tumor or cell population injected therefore probably included fully viable cells as well as those undergoing progressive apoptosis and loss of viability analogous to the effect of VP-16 when it is used to treat a population of tumor cells in vivo.

In further support of this hypothesis is the observation that exposure of normal mice to sublethal numbers of wild-type 3LL cells failed to permit rejection of a subsequent 3LL challenge due to possible pre-existing immunity. In additional control experiments, 3LL challenge of mice previously inoculated with 10^3 3LL cells surviving over 60 days without treatment was uniformly fatal compared to 3LL challenge of mice immunized with 3LL-VP cells ($\chi^2 = 5.0$, $P < 0.0.5$). This also suggests that a potential three-log reduction from 10^6 to 10^3 3LL cells by successful treatment with VP-16 would be insufficient to permit rejection of a subsequent challenge with 3LL unless tumor antigenicity had been augmented by in vivo exposure to VP-16.

VP-16 is a cytotoxic agent known to inhibit topoisomerase II, impair the integrity of DNA strand replication and promote tumor cell apoptosis [3, 9, 10]. There are several mechanisms by which VP-16 might induce immune rejection of 3LL cells. It has been shown that VP-16 markedly increases the gene conversion frequency between MCH class II genes Abk and Ebd, suggesting that the drug can indirectly alter MHC molecules as well as other gene products on tumor cell membranes [8]. The cellular changes due to the apoptotic mechanism of cell death caused by VP-16 may also be involved in the induction of tumor immunity. Albert et al. have shown that human dendritic cells can present antigens from apoptotic influenza A-infected syngeneic or allogeneic cells and can stimulate the induction of class I-restricted CD8 CTL clones independently of endogenous proteosomal pathways [2]. Holmgren et al. have reported that coculture of apoptotic bodies produced by exposure to VP-16 of namalwa cells containing integrated copies of the Epstein-Barr virus-encoded marker, EBNA1-6 gene, results in the expression of EBNA1-6 by phagocytic recipient cells [7]. More recently, it has been reported that treatment of PROb adenocarcinoma-bearing rats with monocyte-derived cells that had phagocytosed apoptotic PROb bodies in the presence of IL-2 causes tumor

regression and long-term tumor protection compared to rats given antigen-presenting cells (APC) that had phagocytosed non-apoptotic tumor cell extracts in the presence of IL-2 [6]. These observations suggest a mechanism whereby APC may acquire antigens from VP-16-treated apoptotic tumor cells for initiation of immune rejection. Other topoisomerase II-inhibiting drugs cause upregulation of the transcription of heat shock protein 70, which can serve as a molecular adjuvant in promoting tumor immunity [11, 12, 19].

Although the initiation of immunity to 3LL probably relates to in vivo processing of 3LL cells undergoing apoptosis following VP-16 treatment, the mechanism(s) responsible for tumor rejection following successful treatment with VP-16 or following inoculation of VP-16-conditioned 3LL is (are) unclear. We were unable to demonstrate CTL-mediated tumor rejection. The excessive binding of serum IgG from tumor-rejecting mice to the crude membranes of 3LL failed to correlate with tumor rejection and appeared to be non-specific. Our current experiments therefore focus on possible cytokine-mediated events and inhibition of angiogenesis in tumor-rejecting mice.

It is increasingly appreciated that the malignant properties of a tumor are a consequence of genetic and/or environmental changes that influence tumor behavior. It has been pointed out that tumor behavior relates to regulatory imbalances rather than autonomy and that "killing strategies may be counter-productive" since they impair the host response and regulatory processes [13]. It is difficult to gauge the extent to which cytotoxic chemotherapeutic drugs undercut the autologous immune response and impair tumor cures in intact hosts. Immunization of patients with genetically modified tumor cells is an active area of investigation and has recently been reviewed by Greten and Jaffee [5]. The use of pharmacologically modified tumor cells to promote tumor immunity has received limited attention. The mutagenic compound, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine has been used by investigators, and produces point mutations in murine P815 mastocytoma. Mutagenized P815 cells are rejected by host mice as well as subsequent challenge of parental mastocytoma [14, 18]. Our work is the first, to our knowledge, to link this effect to a clinically used cytotoxic chemotherapeutic agent.

The pharmacologic concept that cytotoxic drugs produce a constant percentage of tumor cell kill is well established [4, 15]. These drugs are therefore unlikely to eradicate all tumor cells and it is widely felt that cure after effective chemotherapy relates to the host response. Our observations suggest a dual mechanism by which VP-16 promotes cure of cancer in selected patients, i.e. cytotoxic tumor reduction and antigenic modification of tumor cells resulting in immunologic rejection.

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