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The bacterial colicin active against tumor cells *in vitro* and *in vivo* is verotoxin 1

(receptor glycolipid/globotriaosyl ceramide/ovarian cancer/fibrosarcoma/antimetastatic)

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ABSTRACT **We have identified verotoxin 1 (VT1) as the active component within an antineoplastic bacteriocin preparation from *Escherichia coli* HSC_{10} studied over two decades. Recombinant VT1 can simulate the toxicity of anticancer proteins (ACP), and the antineoplastic activity of ACP (and VT1) was abrogated by treatment with anti-VT1 antibody. Similarly, VT1 mimics the protective effect of ACP in a murine metastatic fibrosarcoma model. Prior immunization with VT1 B subunit prevents the effect of VT1 or ACP in this model. The activity of ACP against a variety of human ovarian cell lines was mimicked by VT1, and multidrug-resistant variants were significantly hypersensitive. Primary ovarian tumors and metastases contain elevated levels of globotriaosylceramide compared with normal ovaries, and overlay of frozen tumor sections showed selective VT binding to tumor tissue and the lumen of invading blood vessels. Our contention that VT1 could provide an additional approach to the management of certain human neoplasms is discussed.**

Bacteriocins are bacterial proteins produced to prevent the growth of competing microorganisms in a particular biological niche. A preparation of bacteriocin from a strain of *Escherichia coli* (HSC_{10}) has long been shown to have antineoplastic activity against a variety of human tumor cell lines *in vitro* (1–3) and more recently against *Mycosis fungoides* skin tumors in man (R. Schachter, personal communication). This preparation of partially purified bacteriocin (PPB) or anticancer proteins (ACP) (3) was also effective in a murine sarcoma tumor model to prevent metastases to the lung from an intravenous injection (3).

We now demonstrate that verotoxin 1 (VT1) is the active component within the ACP preparation and that purified VT1 has a potent antineoplastic effect *in vitro* and *in vivo*.

VTs are a family of potent (4) subunit toxins, elaborated by some *E. coli* strains (5), which are involved in the microangiopathy of hemolytic uremic syndrome (HUS) (5–7) and hemorrhagic colitis (HC) (8). Receptor-mediated endocytosis of VT (9–11) is mediated via the binding of the B subunit to the glycolipid globotriaosylceramide [Gal(α1-4)Gal(β1-4)Glc ceramide (Gb_3)] (12–14). The A subunit directly inhibits protein synthesis (15); however, both cytotoxic specificity and specific activity are functions of the B subunit (4). The feasibility of therapeutic use of VT in human neoplastic disease is discussed.

MATERIALS AND METHODS

Cytotoxicity. Recombinant VT1 was purified from pJLB28 (16) as described (4). ACP was prepared from the mitomycin C-treated *E. coli* HSC_{10} by French press disruption and ammonium sulfate precipitation (1–3). Cytotoxicity was measured as described (9). Monoclonal anti-VT1 (13C4) (17), anti-VT2 (18), rabbit polyclonal anti-VT2c (19), and anti-VT1 B subunit (20) antibodies were as described.

Western Blots. Aliquots of ACP preparations and VT1 standard were separated by SDS/PAGE (21), transferred to nitrocellulose, and immunostained (22).

Cell Culture. Cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. SKOVLB and SKOVLC, multidrug-resistant (MDR) variants of the parental SKOV3 cell line (23), were grown with 1 μg of vinblastine or 0.1 μg of vincristine per ml, respectively.

Glycolipid Analysis. Cultured cells or tumor tissue were homogenized in a minimum volume of water and extracted with chloroform/methanol and any Gb_3 present in the lower phase was detected by VT1 TLC overlay (24, 25).

Mouse KHT Fibrosarcoma Metastasis Model. Growth of lung nodules was initiated by injecting 2×10^3 KHT cells mixed with 2×10^6 plastic microspheres (3). The mice were injected i.p. with ACP or VT1 1 day later. Prior i.p. immunization with the B subunit of VT1 was also performed. Lung tumor nodules were counted 18–21 days after cell innoculation.

Toxin Staining of Frozen Tumor Sections. Primary ovarian tumor samples were snap frozen in liquid nitrogen, and 5-μm cryosections were stained with fluorescein isothiocyanate (FITC)-conjugated VT1 (9) as described (26).

RESULTS

Anti-VT1 Neutralizes ACP Cytotoxicity. ACP was cytotoxic for many cell lines, including vero cells, routinely used for the assay of VT cytotoxicity (5). The median cytotoxicity dose (CD_{50}) was $\approx$5 ng of protein per ml. ACP cytotoxicity to KHT cells and for vero cells (data not shown) was neutralized by anti-VT1 but not by anti-VT2 (Fig. 1). By comparison, we estimate the ACP preparation contained $\approx$0.5% VT1.

VT1 Mimics the Antineoplastic Effect of ACP *in Vivo*. Purified VT1 was found to mimic the antimetastatic effect of ACP (3) on the growth of KHT tumor in the lungs of mice (Table 1). Moreover, prior immunization with the VT1 B subunit prevented any protective effect of ACP or VT1 (Table 2). Higher (>100-fold) concentrations of the VT1 B subunit were required to observe activity *in vivo* (Table 2).

ACP Contains VT1. Western blots of ACP demonstrated the presence of VT1 (Fig. 2*A*). By using TLC overlay, anti-VT1 B detected extensive binding of a component within the ACP preparation to Gb_3 and galabiosylceramide (Fig. 2*B*, lane 1). This binding specificity is identical to that of VT1 (12). No

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Abbreviations: VT1, verotoxin 1; ACP, anticancer proteins; MDR, multidrug resistance; FITC, fluorescein isothiocyanate; HUS, hemolytic uremic syndrome; Gb_3, globotriaosylceramide.

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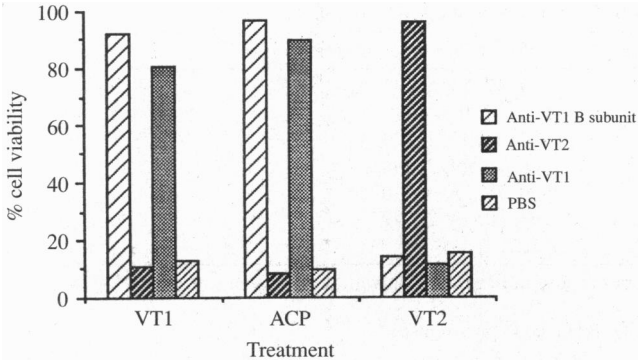

FIG. 1. Neutralization of ACP cytotoxicity by anti-VT. KHT cell monolayers were incubated with increasing dilutions of ACP preparations (2.2×10^5 median cytotoxic doses per mg of protein per ml) from *E. coli* HSC_{10}, VT1, or VT2 in the presence of monoclonal anti-VT1 (13C4) (17), anti-VT2 (18), or polyclonal rabbit anti-VT1 B subunit (20). The cells were cultured for 72 h and viable cells were detected by fixation and staining with crystal violet. PBS, phosphate-buffered saline.

binding component reactive with anti-VT2c was detected (Fig. 2*B*, lane 2).

VT1 Receptor Glycolipid in Ovarian Tumor Cell Lines and Tumors. ACP was active against a variety of ovarian carcinoma cell lines *in vitro* and a comparable dose of VT1 demonstrated similar *in vitro* activity (Fig. 3*A*). By comparison, three of four breast tumor cell lines tested were insensitive, correlating with the presence of Gb_3 (Fig. 4). Interestingly, MDR variants of ovarian tumor cell lines were routinely markedly (≈1000-fold) more sensitive to VT1 (or VT2 or VT2c; data not shown) *in vitro* than the drug-sensitive parental cell line (Fig. 3*B*) and contained more Gb_3 (Fig. 4).

VT1 TLC overlay of extracts of clinical samples (Fig. 5) showed that Gb_3 was absent or barely detectable in normal ovary tissue (4 of 5), whereas in most (8 of 10) ovarian cancer cases, a significant increase in expression of Gb_3 was observed. For one Gb_3 negative ovarian tumor, the metastasis to the omentum was Gb_3 positive (Fig. 5, lanes 20 and 21), as were other omentum metastases (Fig. 5, lanes 36 and 42). The omentum itself was Gb_3 negative (Fig. 5, lane 18). Elevated levels of Gb_3 were found in cells grown *in vitro* from ovarian ascites tumor (data not shown). Ovarian cysts were found to be highly variable in their Gb_3 content, from zero (Fig. 5, lane 11) to over 0.2 nmol/mg of tissue (Fig. 5, lanes 10 and 41). A breast tumor that had metastasized to the ovary was weakly Gb_3 positive (Fig. 5, lane 27).

Tumor cells in frozen sections of ovarian tumor tissue were extensively stained with FITC-VT1 (Fig. 6). Staining of the glandular epithelial tumor is clearly distinguished. The lumen (presumably endothelial cells) of blood vessels within the tumor mass was also strongly stained. No staining of frozen sections of normal ovarian tissue was observed.

DISCUSSION

The similarity of the action of ACP and VT1 on tumor cells both in culture and in animal models and the inhibition by anti-VT1 clearly demonstrate that the toxicity of ACP as described (refs. 3 and 28 and references therein) is due to the presence of VT1. ACP cytotoxicity was neutralized by anti-VT1 but not anti-VT2. Only VT1 was detected by Western blotting (Fig. 2*A*) or glycolipid receptor binding (Fig. 2*B*). Purified VT1 mimicked the ability of ACP to prevent KHT lung metastases from a primary injection (Table 1). Prior immunization with the VT1 B subunit removes this protective effect, both for VT1 and for ACP (Table 2). This conclusively identifies VT1 as the antineoplastic component within the ACP preparation. Moreover, elevated Gb_3 is a metastatic marker for this cell line (29).

Ovarian tumor cell line sensitivity to ACP correlated with sensitivity to VT1 (Fig. 3) and the level of Gb_3 (data not shown). Breast cancer cell lines were, for the most part, toxin resistant (Fig. 3*A*), but one cell line (231) sensitive to ACP was highly sensitive to VT1 and contained Gb_3 (Fig. 4). The MDR variants (SKOVLB, SKOVLC) of the SKOV3 ovarian tumor line, selected for resistance to vinblastine and vincristine, respectively, are >1000-fold more sensitive than the parental cell line (Fig. 3) and contain slightly elevated levels of Gb_3 (Fig. 4). At 10- to 100-fold higher concentrations than the holotoxin, the B subunit alone has been found to induce apoptosis in Gb_3-containing B-cell lymphomas (30). At similar concentrations, the B subunit was also toxic for VT-sensitive, ovarian cell lines (data not shown).

Surgically removed primary ovarian tumor tissue contained increased levels of Gb_3 (Fig. 5) relative to normal ovaries. Gb_3 was found in 8 of 10 primary tumors tested. One of the Gb_3-negative tumors had metastasized to the omentum and was now Gb_3 positive. Ovarian metastases showed significant levels of Gb_3. Toxin overlay of ovarian tumor sections indicates that both the gland-like tumor and the tumor vasculature are reactive (Fig. 6).

Elevated levels of Gb_3 have been associated with other human tumors (31–33), but ovarian tumors have not been previously investigated. Gb_3 is the P^K blood group antigen (34). Tissue surveys using anti-P^K antisera have shown that human

Table 1. Response of KHT cells growing as lung nodules to treatment with VT1 or ACP

Treatment*	No. of mice	No. of lung nodules per mouse†	Mean
Exp. 1			
Control	9	34, 24, 39, 47, 28, 32, 26, 29, 34	32.6
ACP; 0.25 μg per mouse	4	12, 31, 25, 15	20.8
ACP; 1.0 μg per mouse	5	1, 2, 2, 5, 1	2.2
ACP; 4 μg per mouse	5	0, 0, 0, 0, 0	0
VT1; 0.009 μg per mouse	5	29, 41, 34, 29, 21	30.8
VT1; 0.036 μg per mouse	5	7, 16, 29, 16, 6	14.8
VT1; 0.144 μg per mouse	5	1, 4, 2, 3, 1	2.2
Exp. 2			
Control	4	15, 12, 8, 12	11.75
ACP; 2 μg per mouse	5	0, 1, 0, 0, 0	0.2
VT1; 0.1 μg per mouse	2	0, 0	0
VT1B; 0.2 μg per mouse	5	13, 14, 9, 7, 19	12.4
VT1B; 10 μg per mouse	4	8, 3, 9, 11	6.8

*Mice were treated with VT1 or ACP 1 day after cell injection (1000 KHT cells per mouse).
†Lung nodules were counted 20 days after cell injection.

Table 2. Response of KHT lung nodules growing in immunized mice to treatment with VT1 or ACP

Immunization*	Treatment†	No. of mice	No. of lung nodules per mouse‡	Mean
None	None	6	34, 47, 53, 62, 43, 52	48.5
None	ACP; 2.0 μg per mouse	5	0, 1, 2, 0, 0	0.6
VT1B + FA	None	5	43, 40, 47, 43, 23	39.2
VT1B + FA	VT1; 0.2 μg per mouse	6	26, 44, 49, 21, 43, 37	36.7
VT1B + FA	ACP; 2.0 μg per mouse	6	50, 38, 33, 41, 48, 50	43.3
FA only	None	5	44, 60, 19, 25, 40	37.6
FA only	ACP; 2.0 μg per mouse	5	1, 1, 2, 1, 0	1

*Immunization was two injections of VT1 B subunit (VT1B) [10 μg per mouse in Freund's adjuvant (FA)] given (i.p.) 4 weeks and 2 weeks before cell injection.
†Mice were treated with VT1 or ACP 1 day after cell injection (1000 KHT cells per mouse).
‡Lung nodules were counted 20 days after cell injection.

ovaries do not express this glycolipid (35). Our data are consistent with this finding: low levels of Gb_3 were detected in some "normal" ovarian tissue, but the levels were markedly elevated in the ovarian tumor tissue samples. Gb_3 expression in ovarian cysts showed a wide range, which may provide a marker for categorizing cystic ovarian tissue.

Cells at the G_1/S boundary of the cell cycle are particularly VT sensitive, while stationary-phase cell cultures are refractory (36). Ligation of Gb_3 has been shown to induce apoptosis (30, 37). These findings are consistent with a role for Gb_3 in growth control. On VT ligation, Gb_3 has been shown to undergo intracellular "retrograde transport" via the Golgi and rough endoplasmic reticulum to the nuclear membrane (9, 11, 38). Such a pathway may provide the role Gb_3 plays in B-lymphocyte signal transduction (30, 39–42). IgG-committed B cells are sensitive to VT *in vitro* (39), and thus verotoxemia might result in the temporary partial compromise of the humoral immune response.

Prognosis for ovarian cancer is poor (43). VT is considered the cause of potentially fatal human microvascular disease (5). This might be considered to preclude the therapeutic use of VT; however, such a conclusion may be premature.

VTs have been strongly implicated as the etiological agents for HUS (5, 6) and hemorrhagic colitis (8, 44, 45), microangiopathies of glomerular or gastrointestinal capillaries, respectively. Human adult renal endothelial cells are exquisitely sensitive to VT *in vitro* and express a high level of Gb_3 (46). However, HUS is primarily a disease of children under three

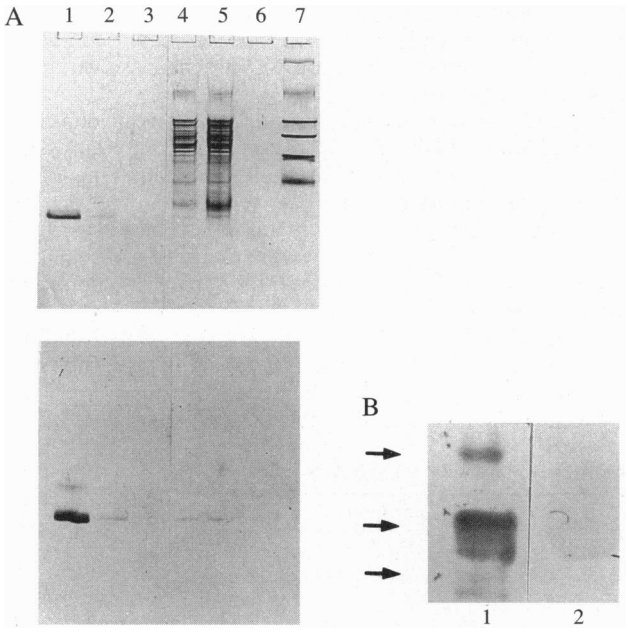

FIG. 2. Detection of VT1 in ACP preparation. (*A*) Western blot. Two ACP preparations and VT1 B subunit standards were separated by SDS/PAGE (21), transferred to nitrocellulose (27), and stained with rabbit anti-VT1 B subunit antibodies described in the *Materials and Methods*. (*Upper*) Coomassie blue stain for protein. (*Lower*) Immunostaining. Lane 1, 1 μg of VT1 B; lane 2, 0.1 μg of VT1 B; lane 3, 0.01 μg of VT1 B; lane 4, ACP preparation 1; lane 5, ACP preparation 2; lane 6, blank; and lane 7, molecular weight standards. (*B*) TLC overlay. ACP was tested for glycolipid binding by TLC overlay (24). Arrows indicate positions of glycolipid standards (from the top) galabiosylceramide, globotriaosylceramide, and globotetraosylceramide. Lane 1, detection with monoclonal anti-VT1; lane 2, detection with rabbit polyclonal anti-VT2c.

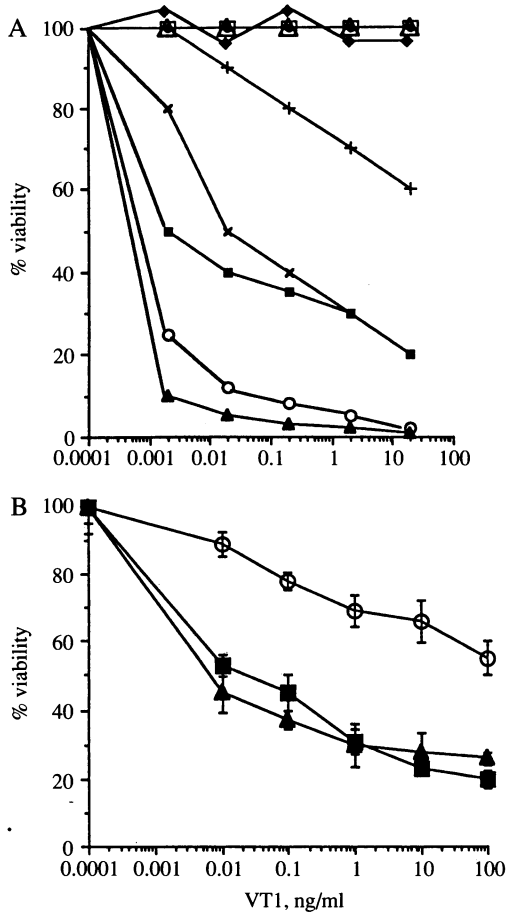

FIG. 3. VT cytotoxicity to ACP-sensitive cell lines. (*A*) Human ovarian and breast tumor-derived cell lines were tested for VT1 sensitivity. Ovarian lines: 1, □; 2, +; 3, ×; 4, ■; and 7, ○. Breast lines: SKBR3, △; 468, ◆; 453, ●; and 231, ▲. The ACP-resistant cell lines 1, 453, and SKBR3 were also resistant to up to 20 ng of VT1 per ml. (*B*) Sensitivity of the ovarian tumor cell line SKOV3 (○) to VT1 was compared with that of MDR variants SKOVLB (▲) and SKOVLC (■).

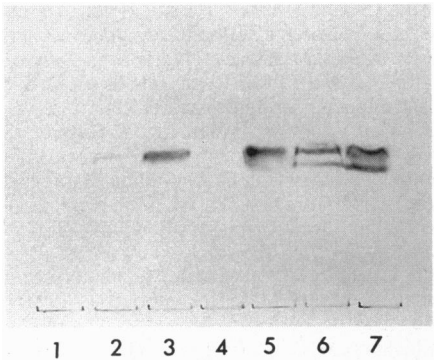

FIG. 4. VT TLC overlay of cell line glycolipids. Glycolipids from an equal number of cells (1×10^6) were extracted and separated by TLC prior to toxin binding. Lane 1, SKBR3; lane 2, 468; lane 3, 231; lane 4, 453; lane 5, Gb_3 standard; lane 6, SKOV3; and lane 7, SKVLB. Cell lines SKBR3, 468, 231, and 453 are derived from breast tumors. Only line 231 is sensitive to VT1.

and the elderly (47–49). Receptors for VT are present in the glomeruli of infants but are not expressed in the glomeruli of normal adults of any age (26). Endothelial cells *in vitro* can be

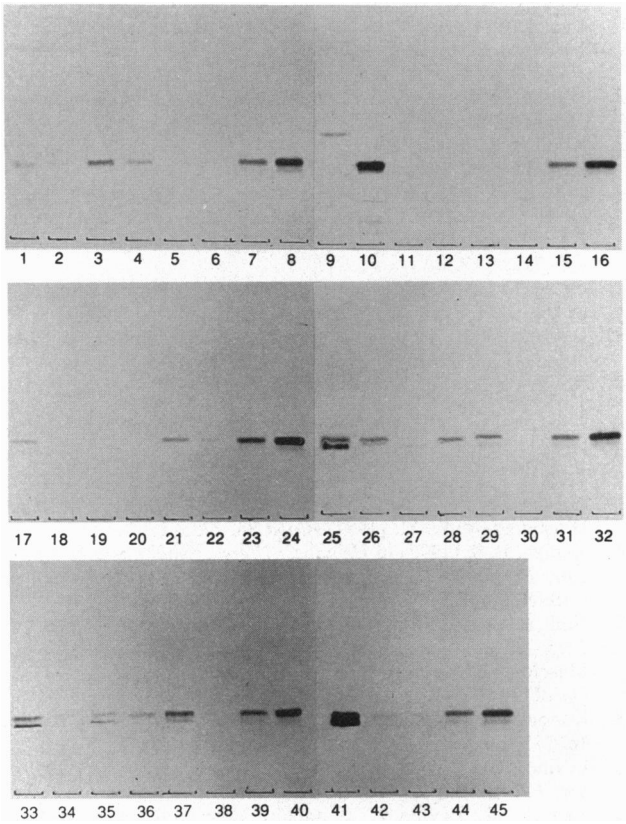

FIG. 5. VT1 TLC overlay of ovary and ovarian-tumor glycolipids. Glycolipids from the equivalent of 10 mg of tissue were separated on a silica column and assayed by VT1 TLC overlay. Human renal Gb_3 standards (0.1, 0.5, and 1 nmol) were run in lanes 6–8, 14–16, 22–24, 30–32, 38–40, and 43–45 to ensure valid comparisons. Lanes 1–5, ovary; lane 9, ovarian cyst; lane 10, ovarian cyst; [lane 11, ovarian cyst; lane 12, ovary; lane 13, peritoneum/bladder]; lane 17, ovarian tumor; lane 18, omentum; lane 19, fallopian/ovarian tumor; [lane 20, ovarian tumor; lane 21, metastasis to omentum]; [lane 25, ovarian tumor; lane 26, ovarian cyst]; lane 27, ovarian tumor (metastasis from breast); lane 28, ovarian tumor; lane 29, ovarian tumor; lane 33, ovarian tumor, [lane 34, ovarian tumor (left); lane 35, ovarian tumor (right); lane 36, metastasis to omentum]; lane 37, ovarian tumor; [lane 41, ovarian cyst; lane 42, omentum metastasis]. Bracketed samples are from the same patient.

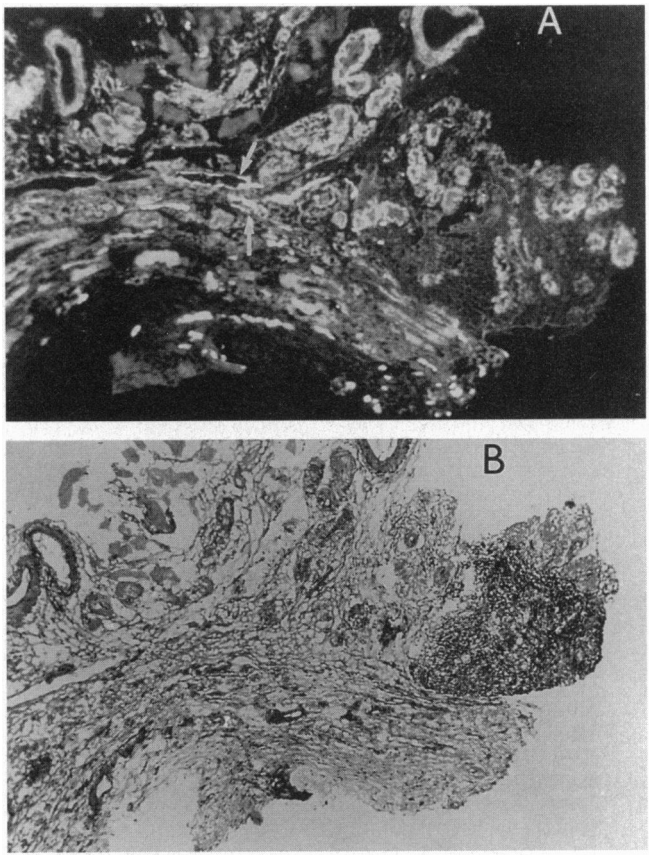

FIG. 6. FITC-VT1 overlay of ovarian tumor frozen section. (*A*) FITC-VT1 staining of tumor. Lumenal staining of blood vessels is marked by arrows. (*B*) Corresponding hematoxylin/eosin stained section. (*A* and *B*, ×17.5.)

sensitized to VT by pretreatment with cytokines (50), resulting in a specific elevation of Gb_3 (46, 51). We have suggested that the transition from intact renal tissue to primary endothelial cell culture results in the maximal stimulation of Gb_3 synthesis from a zero background (26). HUS in the elderly may be the result of two factors: (*i*) verotoxemia and (*ii*) a concomitant stimulation of renal endothelial cell Gb_3 synthesis by some other factor—e.g., lipopolysaccharide stimulation of serum tumor necrosis factor α. Gastrointestinal VT-producing *E. coli* infection does not result in HUS in the general adult population (52), although exceptions have been reported (53). Thus, under normal conditions, the majority of adults—i.e., excluding the extremes of age—should not be liable to VT-induced renal pathology following systemic verotoxemia. This potential "window of opportunity" for VT therapy would include many ovarian cancer patients.

ACP was originally prepared from an *E. coli* strain HSC_{10} (1, 54), and we have now shown the antineoplastic activity is due to the presence of VT1. In hindsight, this is consistent with work by Konowalchuk *et al.* (55) describing VT, since HSC_{10} was one of the few *E. coli* isolates originally demonstrated to produce this toxin.

Tumor regression reported (56) subsequent to the induction of anti-Tj^a antibodies (anti-P^K, -P, and -P1) (12, 57) following incompatible P blood group blood transfusion and VT1 tumor targeting may share a common basis. Our studies suggest that VT could be used to augment conventional drug therapy in adults to treat Gb_3 containing tumors. Preliminary clinical trials using ACP/VT1 injected directly into skin malignancies (*Mycosis fungoides*) have proven successful without adverse systemic effects (R. Schacter, personal communication).

This manuscript is dedicated to the memory of Dr. Farkas-Himsley who died of ovarian cancer during the course of this work. The excellent technical assistance of Ms. Beth Binnington-Boyd and Mr. Robert Kuba is gratefully acknowledged. We are grateful to Dr. W. Chapman (Ontario Cancer Institute) for assistance with the histological evaluation of ovarian tumor sections. The ovarian and breast tumor cell lines were kindly provided by Dr. G. Mills (Toronto Hospital), and the MDR and parental cell lines were a generous gift from Dr. V. Ling (Ontario Cancer Institute). This work was supported by Medical Research Council Program Grant PG 11123 (to C.A.L.) and a Universities Research Initiative grant from the Ontario Ministry of Colleges and Universities (to H.F-H. and R.H.).

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