of vertebrates. It appears that the mechanisms for the propagation of contractions in the alimentary canal in *B. sowerbyi* and vertebrates may be analogous. From a comparative standpoint, some parts of the direct communications between the longitudinal and circular muscle layers in the relatively simple alimentary canal of the worm described here may be replaced by the connective tissue cell bridge in the highly developed alimentary canal of mammals.

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Enhanced expression of Epstein-Barr virus early antigens by antitubulin agents in a latently infected human lymphoblastoid cell line[1]

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Summary. 28 anticancer agents have been surveyed for Epstein-Barr virus (EBV) activating potency. Two vinca alkaloids with antitubulin activity, vinblastine (VLB) and vincristine (VCR), enhanced the expression of EBV early antigens (EA) in a latently infected human lymphoblastoid cell line (Raji), when used in combination with *n*-butyrate. Other antitubulin agents, colchicine, colcemid, and podophyllotoxin, had the same effect, although their effects were less than that of the potent tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA).
Key words. Epstein-Barr virus; tumor promoter; antitubulin agents; vinblastine; vincristine; anticancer agents.

The idea that neoplastic development consists of at least two qualitatively different stages has gained general acceptance. The second, promotion stage has been suggested to be more critical in the development of human cancer than the first, initiation stage (reviewed in Sugimura[3]). Most tumor promoters do not bind DNA and are negative in the Ames mutagenicity assay, whereas initiating carcinogens usually meet one or both of these criteria. Recently, TPA and only those other phorbol esters with tumor promoting activity have been found to induce the viral cycle in latently infected EBV genome carrying cells[4], and we have suggested that a system consisting of EBV nonproducer Raji cells and a low concentration of *n*-butyrate might be useful as a practical screening method for certain types of tumor promoter in our environment[5]. Since many widely used anticancer agents, such as chlorambucil, cyclophosphamide, and procarbazine are carcinogenic in experimental animals, and some are possibly tumor promoters[6], we surveyed 28 anticancer agents for the EBV-EA inducing activity and found enhancement of their expression by two vinca alkaloids as well as other antitubulin agents.

Materials and methods. The human lymphoblastoid cell line Raji, carrying the EBV genome was cultivated in RPMI 1640 medium as described previously[5]. The spontaneous induction rate of EBV early antigens in our subline was less than 0.01%. To assure optimal conditions during the experiment, the viability of the cells was checked both before and after treatment, using the dye exclusion test.

The sources of anticancer agents included Shionogi Co. and Takeda Chemical Indust., Osaka; Nihon Shinyaku, Kyoto; Bristol-Banyu Pharm. Co., Chugai Pharm. Co., Kyorin Yakuhin, Kyowa Hakko Kogyo, Meiji Seika, Nihon Kayaku, Nihon Roche, Sankyo, Taiho Yakuhin Kogyo, and Yamanouchi Pharm. Co., Tokyo, Japan. TPA was obtained from Chemical Carcinogenesis Inc., USA. The *n*-butyrate, colchicine and colcemid were purchased from Nakarai Chemicals Kyoto, Japan. Griseofulvin and γ-lumicolchicine were from Sigma Chemical Co., and podophyllotoxin was from Aldrich Chemical Co., Inc., USA.

The test substances were dissolved either in water, ethanol or dimethyl sulfoxide and assayed for EBV-EA inducing activity as previously described[5]. Briefly, Raji cells were incubated for 48 h with both 4 mM *n*-butyrate and the test substance, and

Table 1. EBV-activating potency of anticancer agents

Anticancer agent*	EBV-EA positive, cells (%)**	Anticancer agent*	EBV-EA positive cells (%)**
Actinomycin	1.2	GE132	1.2
Adriamycin	0.1	Methotrexate	0.8
5-Azacytidine	1.2	Mithramycin	1.0
Bleomycin	1.2	Mitomycin	0.7
Busulfan	0.5	Neocarzinostatin	1.2
Chlorambucil	0.5	Nimustine	
Chromomycin A3	0.5	hydrochloride	1.0
Cis-platinum		OK432	1.2
diammine dichloride	1.2	Pepromycin	1.2
Cytarabine	0.5	PSK	1.2
Daunomycin	1.3	Streptozotocin	1.2
Diethylstilbestrol		Vinblastine	12.0
diphosphate	1.4	Vincristine	13.1
Ftrafur	1.2	VP-16	1.0
5-Fluorouracil	1.2	Control***	2.0

* The anticancer agents were tested at the concentrations of 0.1, 1, 10, 100 and 500 μg/ml. ** Raji cells were incubated with 4 mM *n*-butyrate and various concentrations of test substance for 48 h. Only the maximum values observed for each agent are shown. Values are means of at least two cultures. *** 4 mM *n*-butyrate alone. Values were mostly between 0.1 and 0.2.

Table 2. EBV-activating potency of antitubulin agents

Sample	Antitubulin Activity*	% EBV-EA positive cells Concentration of sample (μg/ml) 100	10	1	0.1	Control TPA**	None***
Vinblastine	+	0.1	12.0	11.2	5.4	29.5	1.4
Vincristine	+	0.1	11.0	13.1	5.6	30.0	2.0
VP-16	−	< 0.1	< 0.1	0.5	0.5	32.0	0.8
Griseofluvin	+	1.2	1.8	1.4	1.7	23.0	1.2
Colchicine	+	0.8	5.8	10.0	8.4	23.0	1.2
γ-Lumicolchicine	−	0.2	0.2	0.2	1.1	31.5	0.2
Colcemid	+	< 0.1	< 0.1	5.2	2.4	28.9	1.3
Podophyllotoxin	+	0.9	0.2	4.3	15.5	30.9	0.2

* See Dustin[7] and Loike et al.[8]. + and − indicate presence and absence of antitubulin activity, respectively. ** Positive control: TPA (20 ng/ml) and *n*-butyrate (4 mM). *** Negative control: *n*-butyrate (4 mM) only.

EA-expressing cells were stained using an indirect immunofluorescence technique and high titer EBV-positive sera from nasopharyngeal carcinoma patients. All experiments were repeated at least twice with controls, and in each assay, at least 300 cells were counted.

Results and discussion. In our system, 4 mM *n*-butyrate alone (negative controls) induced 0.1–2.0% EA-positive cells, and its combination with 20 ng/ml TPA (positive controls) induced 18–35% EA-positive cells. Therefore only those substances which induced more than 5% EA-positive cells were considered active. Of 28 anticancer agents screened, two vinca alkaloids, VLB and VCR, were found to be active; other agents never induced more than twice as many EA-positive cells as those in negative controls (table 1). Although the inducibility of the cells varied from experiment to experiment, the results were reproducible and table 2 shows the result of a typical experiment. It is noteworthy that the optimal concentrations for VLB or VCR were much higher than that of TPA, and that their maximum rate of EA-induction was only a half compared with TPA. Neither VLB nor VCR induced EBV-EA when added to the culture medium without *n*-butyrate (data not shown).

Since both VLB and VCR are known to disrupt microtubules (reviewed in Dustin[7]), other antitubulin agents and related compounds were assayed for their EA-inducibility. As shown in table 2, all antitubulin agents except griseofulvin gave positive results, while γ-lumicolchicine and VP-16, which are derivatives of colchicine and podophyllotoxin, respectively, and have no effect on depolymerizing microtubules[7,8], did not. The fact that EA-induction was observed in the absence of decreased viability compared with controls negates the possibility that it resulted from the selection of EA-expressing cells (data not shown).

Cytoplasmic microtubules have long been implicated in a variety of cell functions such as the maintenance of cell shape, chromosomal movement and the regulation of various plasma membrane phenomena[7]. Antitubulin agents will disrupt these functions as well as the normal linkages between microtubules and intermediate filaments[9]. They may also rupture or disorder the cell membrane of EBV genome-carrying lymphoblastoid cells by altering the structure of cell surface tubulins[10]. TPA, a potent promoter of skin carcinogenesis, induces a wide variety of biological responses in many different cells and tissues, including the cell membrane and chromosomes[11]. Thus, actions of tumor promoters have several aspects in common with those of the antitubulin agents and both have been shown to increase the frequency of viral transformation[12].

The synthesis of viral antigens in latently infected human lymphoblastoid cells has been induced by a variety of conditions and chemicals including TPA, *n*-butyrate, 5-iododeoxyuridine[13], anti-IgM[14], a serum factor[15] and corticosteroids[16]. By combining these inducers and observing additive, synergistic or antagonistic effects on the EBV-EA expression in the Burkitt lymphoma line Daudi, the qualitative differences among them have been revealed[15]. For example, TPA, which has no inducing effect by itself in the Daudi cell, inhibits EBV-induction by corticosteroids but has synergistic, retinoic acid-sensitive effects when combined with *n*-butyrate or the serum factor[16]. The difference in the EBV-EA-inducing potency observed between the antitubulin agent and TPA was probably due to a difference in the underlying mechanisms, which was further suggested by the inability of the former to induce virus replication in the EBV-producer cell line P3HR-1 (J. Fujita, unpublished observation). We and others have shown that the EBV activating agents have considerable overlap with the tumor promoters in their biological activities, and phorbol esters, indole alkaloids and polyacetates have since been demonstrated to have both activities[5,17]. Therefore, it will be important to determine whether these antitubulin agents promote carcinogenesis in experimental animals or whether there is a high risk of primary of secondary cancer following exposure to them.

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