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Effect of (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine on the Growth and Viral Capsid Antigen Expression of Epstein–Barr Virus-Associated Tumor (B-95-8) Cells Transplanted to Nude Mice (42051)

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Abstract. When persistently Epstein–Barr virus (EBV)-infected lymphoblastoid (B-95-8) cells were transplanted subcutaneously or intracerebrally to nude mice of either BALB/c or NIH background, tumors developed, and the tumor cells spontaneously expressed viral capsid antigen (VCA). This model was used to evaluate the *in vivo* anti-EBV activity of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), a highly potent and selective antiherpes agent, which was recently shown to inhibit several parameters of EBV infection *in vitro*. When administered intraperitoneally at 200 mg/kg/day for 4 weeks, or 500 mg/kg/day for 2 weeks, starting immediately after B-95-8 cell inoculation, BVDU effectively reduced tumor growth and VCA expression of either subcutaneously or intracerebrally inoculated B-95-8 cells.

Epstein–Barr virus (EBV) is the causative agent of infectious mononucleosis and is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, and certain immunoblastic sarcomas arising in allograft recipients (1). *In vitro,* EBV may infect epithelial cells (2) as well as B lymphocytes (both lymphoblastoid cell lines and Burkitt lymphoma cells). The latter readily produce tumors at the site of inoculation when transplanted to nude mice (3, 4). Very few agents have been assessed for their inhibitory effects on EBV. Those that have been found effective in inhibiting EBV replication *in vitro* include acyclovir [9-(2-hydroxyethoxymethyl)guanine] (5, 6), phosphonoformate (7), BVDU [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine] (8, 9), FMAU [1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-methyluracil], and FIAC [1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-iodocytosine] (8). While the inhibitory effects of acyclovir on EBV replication were reversible, those of BVDU, FMAU, and FIAC persisted after removal of the drug from the cell culture medium (8). With acyclovir clinical trials have been started in the treatment of infectious mononucleosis (10, 11). Although these trials are still in progress, preliminary analyses of the results indicate that acyclovir may interrupt transiently EBV excretion in the oropharynx (11).

It has been known for some time that EBV-carrying cells (i.e., lymphoblastoid cells), when transplanted to athymic-nude mice, grow up to tumors at the site of inoculation (12). However, no attempts have been made to assess the applicability of this model for chemotherapeutic purposes. These studies would seem warranted in view of the recent demonstration that selected antiherpes agents such as BVDU are rather potent inhibitors of EBV replication *in vitro* (8, 9). BVDU has been found efficacious in a variety of experimental herpes simplex virus type 1 (HSV-1) infections, including HSV-1 skin lesions, HSV-1 keratitis, HSV-1 encephalitis in mice, guinea pigs and rabbits [for a review, see Refs. (13, 14)]. BVDU proved particularly effective in protecting nude mice against the mortality (due to encephalitis) associated with subcutaneous HSV-1 infection (15).

The present study was aimed at (*i*) establishing an animal model for EBV-associated tumors, based on the subcutaneous (sc) or intracerebral (ic) inoculation of persistently EBV-infected (B-95-8) cells into nude mice, and (*ii*) evaluating the inhibitory effects of BVDU on tumor cell growth and virus rep-

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lication in these systems. The inhibitory effect of BVDU on tumor growth was assessed by measuring tumor size (or number of tumor cells) and its inhibitory effect on virus replication was monitored by measuring VCA expression.

Materials and Methods. *Mice.* Female nude (*nu/nu*) mice of BALB/c or NIH background [imported from NIH (U.S.A.) in 1982 and designated NIH *nu/nu*] were supplied by the Animal Centre of the Chinese Academy of Medical Sciences. The mice were bred and kept under standardized conditions in a plastic hood with sterile airflow at a temperature of 25°C and a relative humidity of 80%. For each experiment, a pair of mice was chosen from one litter, and matched so that one partner was used for BVDU treatment and the other as control. The following parameters were used to assess drug toxicity: color and luster of the skin, movements of the mice, and their consumption of water and food.

B-95-8 cell lines. The B-95-8 cell line is a marmoset lymphocyte cell line originally infected with EBV from a patient with infectious mononucleosis (16). The B-95-8 cells were cultured at 37°C in glass prescription bottles in RPMI 1640 medium containing penicillin 100 U/ml and streptomycin 100 μg/ml, and supplemented with 20% newborn calf serum. Prior to transplantation, the cells were enumerated by the trypan blue stain method, and the number of VCA-positive cells was estimated by an immunoenzymatic assay (see below). Before transplantation, the cells were suspended in medium at a concentration of 1×10^8 viable cells/ml.

Procedures: Subcutaneous inoculation of B-95-8 cells. B-95-8 cells were transplanted subcutaneously on the back of BALB/c nude mice at a rate of 2×10^7, 1.5×10^7 or 1.0×10^7 cells in a volume of 0.2 ml per mouse. Biopsy specimens were taken once weekly. Starting immediately after transplantation of the B-95-8 cells, BVDU was administered intraperitoneally at a dose of 200 mg/kg/day (divided over 2 doses a day), for 4 weeks. Control mice received saline.

As a rule, the transplanted cells grew up to a tumor of 5–8 mm in diameter within 3 weeks. Their growth further increased during the next weeks so that the largest tumor was $12 \times 18 \times 25$ mm^3 by the end of the 3-month observation period. By then, the mice were killed and examined macroscopically for metastases in lung, liver, brain, and other organs.

At 1, 2, 3, 4, and 5 weeks after transplantation, when the tumor appeared as a pale ball clearly discernible from the surrounding tissue, tumor fragments were removed and examined for VCA-positive cells by an immunoenzymatic procedure (see below).

During the observation time, the length and width of the tumors were measured weekly by a calipers, and their volume was calculated according to the formula $V = 4/3\ \pi\ L/2\ (W/2)^2$. These measurements were made only for those tumors that developed in mice inoculated with 2×10^7 cells, which had not been treated with BVDU and from which no biopsy specimens had been taken.

Intracerebral inoculation of B-95-8 cells. B-95-8 cells were transplanted intracerebrally to NIH nude mice at a rate of 1.2×10^6 cells in a volume of 0.02 ml. The cells were inoculated with a tuberculin syringe at the right frontal region of the brain at a depth of 2 mm. Starting immediately after transplantation of the cells, BVDU was administered intraperitoneally at a dose of 500 mg/kg/day (divided over 2 doses a day) for 2 weeks. Control mice received saline. At the end of the 2-week period, the mice were killed; the brains were removed, washed several times with saline, and separated with a lancet into two parts, starting from the temporal surface and following the needle mark by which the B-95-8 cells had originally been inoculated. The tumor explant was thereby split into two equal parts. One part was fixed in Bouin's solution for further histopathological examination. The other part of the tumor was transferred to a glass tube containing 3 ml of 0.85% NH_4Cl. A separation of the tumor cells was achieved by several blowings with a pipet. The total number of tumor cells was then enumerated microscopically.

Histopathological examination. For the subcutaneous tumors, biopsy specimens were taken at the third week and autopsy specimens on the day the mice were killed (usually at the fifth or sixth week). Usually, the mouse from which a biopsy specimen was taken at

the first and second week for assaying VCA-positive cells was submitted to the histopathological test at the third week. The mice that were still alive at the tenth week were also used for histopathological examination. Tumor tissue was fixed in Bouin's solution, sliced by a histotome, and stained with hematoxylin. In some instances, the assay for VCA-positive cells was performed on the same cell smears as the histopathological examination. To determine the size of the intracerebral tumors, the tumor fragments were examined microscopically and their surface was measured in square millimeters.

Drug. BVDU was synthesized by R. Busson and H. Vanderhaeghe at the Rega Institute for Medical Research (Leuven), essentially following the procedure described by Jones *et al.* (17). The compound was stored at 4°C.

Immunoenzymatic assay. Indirect immunoenzymatic assay for VCA detection was performed as described previously (18, 19). The cells from biopsy specimen were smeared on glass slides, fixed in acetone for 30 min, and then treated with (EA^+/VCA^+) antiserum from an NPC patient at 37°C for 30 min. After three successive treatments with PBS (pH 7.6) for 5 min each, the cell smears were exposed to anti-human IgG horse IgG; this horse IgG had been previously conjugated with peroxidase. The cells were then washed three times with PBS and exposed to Tris–HCl buffer, pH 7.6, containing 3'3'-diaminodiphenylenediamine and 0.1% H_2O_2. The VCA-positive cells were counted microscopically.

Results. *Growth of B-95-8 cell tumors upon subcutaneous inoculation of B-95-8 cells into BALB/c nude mice.* In the first experiment, tumor development was followed in mice inoculated subcutaneously with B-95-8 cells when 3 weeks old. All mice developed tumors at the inoculation site. Within the first 3 weeks tumor size increased rather slowly, then the pace of tumor development accelerated and by the eighth week tumor size had increased by 300- to 1600-fold (Fig. 1). There were marked differences in the size of the individual tumors; the difference in size between the smallest and largest tumor being about fivefold: 200–1055 mm^3 at the fifth week, and 300–1687 mm^3 at the eighth week.

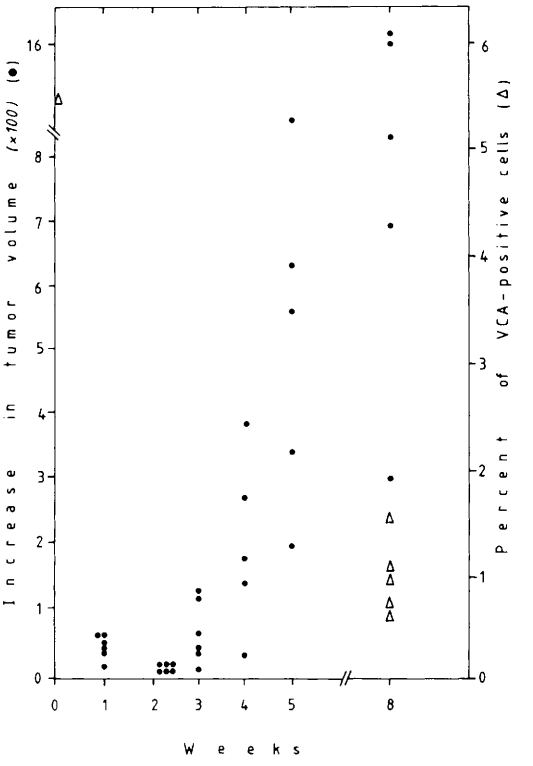

FIG. 1. Tumor development in BALB/c nude mice inoculated subcutaneously when 3 weeks old with 2×10^7 B-95-8 cells. The results are presented for six individual mice. One mouse died between the third and fourth week. The volume of the tumors was estimated as explained under Materials and Methods. The number of VCA-positive cells was determined for the original inoculum and the tumor cells that were harvested after 8 weeks.

The B-95-8 cells which were used for inoculation as well as the tumor cells harvested at the eighth week were examined for their content of VCA-positive cells. For the original cell inoculum, the percentage cells expressing VCA was 5.5%; after 8-week growth in the nude mice, it had declined to 0.7–1.6% (Fig. 1). It was obvious, therefore, that if VCA expression were to be monitored as a parameter of tumor cell development, it had to be measured during the first few weeks after tumor cell inoculation. As shown in Fig. 2, the percentage of VCA-positive cells increased during the first to second week after tumor cell transplantation up to two- to three-fold higher than the percentage of the initial VCA

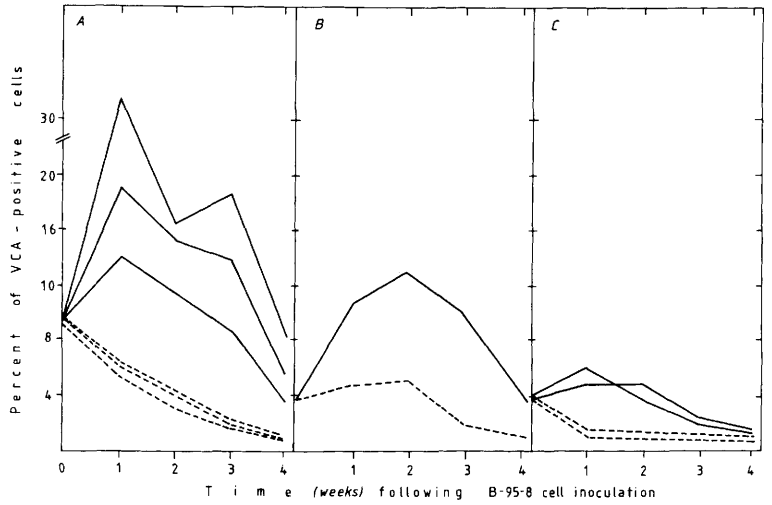

FIG. 2. Effect of BVDU on VCA expression of subcutaneously transplanted B-95-8 cells in 3-week-old BALB/c nude mice. Six pairs of nude mice, matched by sex, age, and litter, were inoculated: three pairs with 2×10^7 cells per mouse (A), one pair with 1.5×10^7 cells per mouse (B), and two pairs with 1×10^7 cells per mouse (C). Immediately after inoculation of the B-95-8 cells, BVDU (- - -) was given intraperitoneally at 200 mg/kg/day to six mice, and their partners were treated with saline (—). Biopsy specimens were taken weekly and assayed for VCA expression.

expression. Afterward, the percentage of VCA expression declined gradually, to return to the original level by the third to fourth week. From these results it is clear that nude mice are able to maintain and support the growth of B-95-8 cells and enable these cells to express VCA. No metastases were observed macroscopically in the liver, lung, kidney, brain, or any other organ.

Effect of BVDU on VCA expression of subcutaneously inoculated B-95-8 cells. Paired mice which had been inoculated subcutaneously with B-95-8 cells (Fig. 2) were treated with either saline or BVDU at 200 mg/kg/day for 14 days. As shown previously (15), this BVDU dosage is perfectly well tolerated by nude mice. Irrespective of the size of the tumor cell inoculum (Figs. 2A, B, C), BVDU treatment caused a marked decrease in the percentage of VCA-expressing cells at either the first, second, third, or fourth week after tumor cell inoculation. This was particularly clear for the mice inoculated with 2×10^7 cells, where BVDU treatment resulted in a three- to sixfold decrease in the number of cells expressing VCA.

Growth of B-95-8 cell tumors upon intracerebral inoculation of B-95-8 cells into NIH nude mice. As originally shown by Epstein *et al.* (20), inoculation into an immunologically privileged site such as the brain may enhance the transplantability of EBV-carrying lymphoid cells. B-95-8 cells were transplanted intracerebrally into NIH nude mice when either 3, 5, or 9 weeks old (Table I); 1.2×10^6 cells per mouse were inoculated. Two weeks after inoculation, the brain was removed and the right hemicerebrum in which the B-95-8 cells had been inoculated was divided into two parts following the needle mark: the first part was used for determination of the number of tumor cells and percentage VCA-positive cells, the second part was submitted to histological examination. It was thereby assumed that the tumors grew as spherical nodules. Two weeks after transplantation into 3-week-old NIH nude mice the number of B-95-8 cells had increased by 25-fold, whereas the percentage of VCA-positive cells slightly raised from 4.2 to 4.7% (Table I). For the mice inoculated at the age of 5 or 9 weeks, the number of B-95-8 cells also increased (by 20-fold and 8-fold, respec-

TABLE I. DEVELOPMENT OF B-95-8 CELL TUMORS IN NIH NUDE MICE FOLLOWING INTRACEREBRAL INOCULATION

	Age of mice at tumor cell inoculation (weeks)		
	3	5	9
Before inoculation			
(*i*) Number of tumor cells per mouse inoculated	1.2×10^6	1.2×10^6	1.2×10^6
(*ii*) VCA-positive cells (%)	4.2	2.2	1.5
Two weeks after inoculation			
(*i*) Number of tumor cells harvested (per mouse)	3.0 ± 0.45 ($\times 10^7$)	2.4 ± 0.9 ($\times 10^7$)	0.9 ± 0.15 ($\times 10^7$)
(*ii*) VCA-positive cells (%)	4.7	1.4	0.3–0.5

Note. The tumor cells harvested 2 weeks after inoculation were characterized as lymphoblastoid cells by histological examination. There were eight mice per group.

tively), but the percentage VCA expression diminished from 2.2 to 1.4 in the 5-week age group and from 1.5 to 0.5 in the 9-week age group.

Effect of BVDU on VCA expression and growth of intracerebrally inoculated B-95-8 cells. NIH nude mice which had been inoculated intracerebrally with B-95-8 cells when 3 weeks old were treated with either saline or BVDU at 500 mg/kg/day for 14 days. No signs of drug toxicity were observed at this dosage regimen. In this experiment tumor size was evaluated by microscopic measurement of the surface of the tumor slices. The number of tumor cells and percentage of VCA expression were determined as described above. Treatment with BVDU resulted in a marked reduction of all three parameters of B-95-8 tumor growth: (*i*) tumor size; (*ii*) number of tumor cells, and (*iii*) VCA expression (Table II).

Discussion. The athymic-nude mouse model represents an invaluable tool for studying tumor heterotransplantation (12) and inhibition of these heterotransplants by antitumor agents (21). Apparently, the nude mouse provides a permissive environment for the growth of EBV-infected cells; and this confirms the work of Povlsen *et al.* (22), Trumper *et al.* (23), and Rabin *et al.* (24). However, Rabin *et al.* (24) did not observe tumor growth upon subcutaneous inoculation of B-95-8 cells in nude mice. This discrepancy with our results may be attributed to a number of factors, i.e., the strain of mice (NIH Swiss nude mice in Rabin's experiments versus BALB/c nude mice in our studies), the age of mice (5 weeks versus 3 weeks) and the number of cells inoculated (3.5×10^6 cells versus $1–2 \times 10^7$ cells).

From a review of the literature (3, 4, 12, 22, 25–27), it is clear that lymphoblastoid cells transplanted into nude mice have thus far not been pursued from a chemotherapeutic viewpoint. We have now established such a model and in this model we have evaluated the antiviral and antitumor potentials of

TABLE II. EFFECT OF BVDU ON GROWTH OF B-95-8 TUMOR CELLS INOCULATED INTRACEREBRALLY IN NIH NUDE MICE WHEN 3 WEEKS OLD

Parameter	Control	BVDU
Tumor size (mm^2)	5.6 ± 0.7	2.97 ± 1.02 ($P < 0.01$)
VCA-positive cells (%)	4.7	1.2
Number of tumor cells harvested (per mouse)	3 ± 0.45 ($\times 10^7$)	1.76 ± 0.43 ($\times 10^7$) ($P < 0.01$)

BVDU, a potent antiherpesvirus drug (13, 14, 27), which inhibits several parameters of EBV infection *in vitro* (9).

When transplanted subcutaneously to 3-week-old nude mice, B-95-8 cells grew up to tumors at the inoculation site. They gradually increased in size during the 8-week observation period. The percentage of VCA-expressing B-95-8 cells increased during the first 2 weeks after transplantation but decreased thereafter, so that 8 weeks after transplantation VCA expression was only one-fifth of the original value. B-95-8 cell tumor growth was also observed after intracerebral inoculation of the cells into 3-week-old nude mice. In this experiment the mice were followed for 2 weeks only; VCA expression remained essentially unaltered during this period, whereas the number of tumor cells increased 25-fold.

Treatment with BVDU at 200–500 mg/kg/day for 2 weeks resulted in a significant reduction in the percentage of VCA-expressing B-95-8 cells, irrespective of the route by which the cells had been inoculated, subcutaneous or intracerebral. Also, BVDU treatment led to a significant reduction in the size of tumors and number of intracerebrally transplanted B-95-8 tumor cells.

The inhibitory effects of BVDU on VCA expression by B-95-8 cell transplants in nude mice seem consistent with its *in vitro* effects on several parameters of EBV infection (9). The minimum concentration of BVDU required to reduce VCA expression of B-95-8 cells *in vitro* is 0.01 m*M* (3 μg/ml) and this concentration may be achieved, even in the brain, upon intraperitoneal administration of BVDU at doses of 200 or 500 mg/kg (28).

The exact mechanism by which BVDU inhibits VCA expression and B-95-8 cell growth *in vivo* remains to be determined. Based on its *in vitro* effects (9), BVDU may be expected to suppress both replication and activation of EBV, and its inhibitory effects on B-95-8 cell growth may be mediated by a selective elimination of productively infected cells (8).

It is unlikely that BVDU would interfere with B-95-8 cell growth or VCA expression through a host cell-mediated response, since BVDU does not interact with normal uninfected host cells, i.e., mitogen- or antigen-stimulated lymphocytes, up to a concentration of approximately 100 μg/ml (29, 30). Furthermore, the inoculation of B-95-8 cells into an immunologically privileged site (brain) and the relative insensitivity of EBV-infected cells to the cytotoxicity of NK cells (31) minimize the possibility of an immunomediated action of BVDU.

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