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Increased Infectivity of Oncogenic Herpes Viruses of Primates with Tumor Promoter 12-*O*-Tetradecanoylphorbol-13-Acetate (40901)[1]

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Abstract. Cell cultures of simian and human origin infected with two strains each of herpesvirus saimiri (HVS) and herpesvirus ateles (HVA) were compared for production of infectious virus and early and late antigens (EA, LA) in the presence and absence of the tumor promoting agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Second, Epstein–Barr virus (EBV) infected human and simian lymphoblastoid cell lines of high and low cell passages were also compared for enhanced production of early antigen and virus capsid antigen (VCA) using two concentrations of TPA, owl monkey kidney (OMK) and squirrel monkey lung. Cell cultures infected with HVS and HVA with 20 ng/ml of TPA exhibited higher percentages of early and late antigen producing cells and contained 1.0–1.6 logs more virus. Such cells also had earlier cytopathic effects, larger plaques, and a 3-fold increase in the number of plaques. The TPA also enhanced HVS-EA, and LA both in OMK and human skin fibroblast (HSF) cells. The enhancement of EA was approximately 17.0% more in OMK cells and 4.0% more in HSF. The HVS-LA was 22% higher in OMK and 6.0% higher in HSF cells. Pretreatment of OMK cells with TPA prior to HVS or HVA infection showed only a 0.5–0.7 log enhancement of virus. A dose–response of TPA in P_3HR1 cells showed that both 20 and 40 ng/ml doses were able to enhance EBV, EA, and VCA significantly with peak enhancement at 40 ng/ml. Higher doses of TPA (60 and 100 ng/ml) resulted in considerable cell death and reduction in antigen production. TPA (20 and 40 ng/ml) stimulated both VCA and EA antigen production in P_3HR1 and B95-8 cells, with lesser effects on other human and simian lymphoblastoid cells. However, the stimulation of EA and VCA with these doses of TPA varied for each cell line. Moreover, TPA-treated P_3HR1, B95-8, and 407-I cells on the average also produced 1.0–1.5 logs more virus than the untreated cells. The higher percentage of EA-VCA production from P_3HR1 and B95-8 cells and lower EA-VCA from other human and simian cells suggests that such virus–cell interaction may be influenced by *in vitro* passages.

Phorbol diesters and some related plant diterpene esters that promote tumors in mouse skin have been shown to exert many biochemical and biological effects on cells in culture (1, 2). Among the phorbol diesters 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is found to be a potent tumor promoter (1, 2) as well as a mitogen (3–7). In addition TPA has been shown to induce early antigen (EA) in primate lymphoblastoid cells carrying herpesvirus genomes and to enhance virus capsid antigen (VCA) in Epstein–Barr virus (EBV)-producing lymphoblastoid cells (8). TPA also increases the efficiency of cell transformation by adenovirus 7 (9) and EBV (3, 10). In this communication we report the interaction of TPA with three primate oncogenic virus–cell systems, i.e., herpesvirus saimiri (HVS), herpesvirus ateles (HVA), and EBV. Continuous cell cultures of simian and human origin, either from high or low *in vitro* cell passages, or producing EA and VCA, were compared for antigen content as well as virus production in the presence of TPA to determine its effect on antigen or virus yields after long term *in vitro* passage.

Materials and methods. Cells. The following simian and human cells were employed for HVS and HVA studies with TPA: a continuous cell line of owl monkey kidney (OMK) supplied by Drs. Melendez and Hunt of the New England Primate

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Center; chimpanzee skin (CS) and human skin (HS) cultures supplied by Drs. Huebner and Rhim of the National Cancer Institute; and squirrel monkey lung (SML) cell culture supplied by Dr. Rangan of the Delta Regional Primate Center.

These cells were propagated in MEM and Dulbecco's modified MEM with 10% heat-inactivated fetal calf serum (△FCS) and regular amounts of antibiotics. After confluent growth the cultures were maintained in 5% △FCS.

To investigate the interaction of EBV and TPA the following human and simian lymphoblastoid cell lines were used: the human lines included P_3HRI, a producer of nontransforming EBV; and recently established EBV producer B-cell lines—Landis from an American Burkitt's tumor, and AG876 from an African Burkitt's tumor. Landis and AG876 cell lines were established and supplied by Drs. Pizzo and Magrath of the National Cancer Institute (11, 12).

Simian EBV cell lines included B95-8, a transforming EBV producer established from a cotton-topped marmoset, and two recently established lymphoblastoid cell lines, 407-I and 731-C, derived from peripheral blood of 7-year-old cotton-topped marmosets by *in vitro* infection with B95-8 EBV in the presence of TPA (3). All simian and human lymphoblastoid cells were grown and maintained in RPMI-1640 and 10% △FCS.

Viruses. HVS prototype strains S295 and S396-0 propagated in OMK cells with titers of $10^{5.5}$ and $10^{4.5}$ $TCID_{50}$/ml, respectively, were used (13). HVA strains 810 and 73 (13) also grown in OMK cells to titers of $10^{3.5}$ and $10^{4.0}$ $TCID_{50}$/ml, respectively, were used. For TPA and virus interactions 100 $TCID_{50}$/ml of HVS-S295, and 1000 $TCID_{50}$/ml of HVS-S396-0, HVA-810 and 73, were used throughout the experiments.

Antigen assays. The assays for EBV-EA and VCA used in our studies have been described by Henle *et al.* (14). The assays for HVS-EA and late antigen (LA) have also been described previously (15, 16).

TPA was obtained from Sigma Chemical Company, St. Louis, Missouri. The 10 mg of TPA was dissolved in 1 ml of DMSO and stored at −70°C. Before use, 100 μl of the stock solution (i.e., 100 μg TPA) was dissolved in 10 ml of serum-free medium. Required amounts of this solution were added to cell cultures. The final DMSO dilution in the cultures was $10^{-5}-10^{-6}$.

Results. Herpesvirus saimiri and herpesvirus ateles. Table I describes the effect of TPA on production of HVS and HVA. Cells infected with HVS and HVA and subsequently treated with TPA showed a significant increase in virus titer in all experiments. Moreover, such cultures also exhibited early CPE. The effect of the TPA was not consistently related to the properties of the virus. Enhancement in OMK cells was more pronounced with HVS-S295, a fast replicating virus strain, than with the more slowly replicating HVS-S396-0 strain (13). HVS-S396-0 and HVA-810 showed higher titers in SML cells (1.5 log) in the presence of TPA. For HVA, however, the more slowly replicating HVA-810 showed greater enhancement with TPA of infectious virus (1.5 log) than was obtained with the faster replicating HVA-73 strain (1.0 log). Treatment of OMK cells with 40 ng/ml of TPA (not reported in Table I) was toxic to cells and enhancement of infectious virus was lower than that obtained by 20 ng/ml. The cells treated with TPA and subsequently infected with HVS and HVA showed minimal increases in viral titers (Table I).

We also studied the enhancement of HVS antigens in the presence of TPA in OMK and HS cells for HVS-EA (Fig. 1). OMK and HS cells infected with 1000 $TCID_{50}$/ml of virus were treated with 100 μg/ml of phosphonoacetic acid (PAA) in the presence and absence of 20 ng/ml of TPA (16). On the average there was 17.0% more HVS-EA producing OMK cells in the presence of TPA. TPA-treated HS cells showed an increase of 4.0% HVS-EA over TPA-untreated cells. For HVS-LA, the cells treated with virus and TPA showed an increase of 22% LA in OMK cells, and for HS cells the increase of LA was 6.0% (Fig. 1). This further suggested that TPA enhanced both EA and LA of HVS.

The CS fibroblast cells infected with 1000 $TCID_{50}$/ml of HVS-S396-0, HVA-810, and

TABLE I. ENHANCEMENT BY TPA OF PRODUCTION OF INFECTIOUS HVS AND HVA FROM OMK AND SML CELL CULTURES

Virus and strain	Cell line	Mean virus titer/ml[a] Untreated (SD)	Mean virus titer/ml[a] TPA treated (SD)	Average log enhancement
TPA treatment was preinfection[b]				
HVS-S295	OMK	5.25 (0.129)	6.0 (0.28)	0.75
HVA-810	OMK	3.5 (0.346)	4.1 (0.141)	0.6
HVA-73	OMK	3.95 (0.173)	4.5 (0.0)	0.55
TPA treatment was postinfection[c]				
HVS-S295	OMK	5.25 (0.129)	6.75 (0.353)	1.5
HVS-S396-0	OMK	4.78 (0.035)	5.85 (0.495)	1.07
HVA-810	OMK	3.5 (0.346)	5.1 (0.141)	1.6
HVA-73	OMK	3.95 (0.173)	5.25 (0.353)	1.3
HVS-S396-0	SML	3.0 (0.0)	4.5 (0.43)	1.5
HVA-810	SML	3.0 (0.42)	4.5 (0.707)	1.5
HVA-73	SML	2.5 (0.424)	3.5 (0.42)	1.0

[a] The titers represent two separate experiments with HVS and HVA, using OMK cells (standard deviation at 95% confidence level is given in parentheses). Four microtissue culture plates were used per dilution. Virus titer is expressed as $\log_{10}$.

[b] Cells at 90% confluency were treated with 20 ng TPA/ml medium for 3 days, washed with serum-free medium, then infected with virus for 1 hr; they were washed again, fed with 5% FCS medium, and incubated at 37°C. The virus was harvested when OMK cells showed 3+ to 4+ CPE. (The reason for using confluent cell monolayer was to provide maximum number of cells for adsorption of TPA.)

[c] Cells were infected with virus when they reached 70–75% confluency. Virus was absorbed for 1 hr at 37°C, then the cells were washed and fed with medium containing 5% ΔFCS. TPA was then added at 20 ng/ml medium. The virus, with and without TPA was titered when all cells showed 3+ and 4+ CPE.

73 for 1 hr and then kept with 20 ng/ml of TPA in medium containing 5% FCS showed more plaques, and such plaques appeared at least 24 hr earlier than those observed in cultures without TPA. The plaques were also at least two to three times larger in size. HVS-S396-0 produced more plaques both in the TPA-treated and control cells than HVA 810 and 73. There was at least a three fold increase in number of plaques with HVS and HVA. OMK cells infected with HVS-S295 and treated with TPA used as a control for the above experiment showed approximately 97 plaques in the TPA-treated OMK cells as compared to 35 in untreated cells. Even though all cell types gave significant increases in plaques with TPA treatment, the infective virus yields (1.0–1.5 log) did not correlate with the number of plaques.

Epstein–Barr virus. A dose-response evaluation of the effect of TPA in stimulating EBV-VCA and EA was done in P_3HR1 cells to select the doses which produced maximum enhancement of EBV antigens with lowest cell death. Peak (45%) antigen production was found to be 40 ng/ml of TPA (Fig. 2). Doses of 60 and 100 ng/ml of TPA reduced the number of antigen-producing cells. In fact, treatment with 100 ng/ml of TPA resulted in 4% less antigen-producing cells than observed in the untreated P_3HR1 cells. These cell viability data support the observation that 60 and 100 ng/ml of TPA are cytotoxic. Untreated P_3HR1 cells had $\geq$75% viability as compared to $\leq$52 and $\leq$40% in 60 and 100 ng/ml TPA-treated cells, respectively. Cell toxicity was further demonstrated by considerable cell debris and nonspecific fluorescence. Treatment of cells with 20 and 40 ng/ml of TPA also resulted in some cell death (viability of $\leq$68 and $\leq$60%, respectively). Based on these data, further experiments with EBV producer cells of human and nonhuman primate origins were performed with 20 and 40 ng/ml doses of TPA.

The experiments performed with producer cell lines of transforming (B95-8, AG, Landis, 407-I, and 731-C) and nontransforming P_3HR1 EBV are presented in Fig. 3. The results show that enhancement

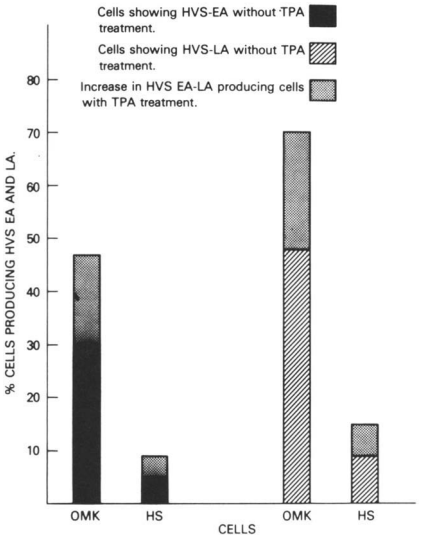

FIG. 1. Comparison of HVS, EA, and LA producing cells, OMK, and human skin fibroblast (HS) in presence and absence of TPA. (1) 20 ng/ml of TPA was used after HVS adsorption. (2) In OMK-infected cells, EA was evaluated when non-PAA-treated cells showed ≥3 + CPE. HS cells were examined for EA when non-PAA-treated cells showed areas of CPE. (3) LA was examined in OMK cells approximately 4–6 days post infection and at this time these cells showed between 3 to 4 + CPE. HS cells were examined when there were areas of CPE in different parts of the monolayer. It generally took 8–12 days to see this effect in HS cells. (4) The antigen producing cells presented here were mean of three experiments.

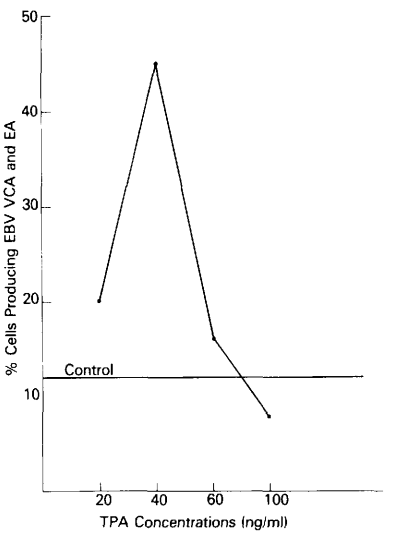

FIG. 2. P_3HR1 cells at ≥85% viability were initially seed at 5×10^5 cells/ml in RPMI-1640 with 10% ΔFCS in presence and absence of various doses of TPA. These cells were examined for cell viability and percentage EBV-VCA and EA 4 days after TPA treatment. (●——●)P_3HR1 cells showing EBV, VCA, and EA in presence of TPA. (——)P_3HR1 cells showing EBV, VCA, and EA without TPA.

of EA-VCA with TPA in human and simian cells varied. The percentage of antigen producing cells was significantly higher in B95-8 and P_3HR1 cells than in other human and simian cell lines at low passage levels. Moreover, treatment of cells with two doses of TPA (Fig. 3) shows that some cell lines respond better with 20 ng/ml (AG, B95-8, 407-I) and others with 40 ng/ml (P_3HR1, Landis, 731-C) in terms of EA-VCA enhancement. Following TPA treatment P_3HR1 cells at 2×10^6 cells/ml concentration and with more than 80% viability had less EA-VCA than 5×10^5 cells/ml with the same viability. The data on EB virus yields from P_3HR1, B95-8, and 407-I showed that on the average TPA-treated cells produced between 1.0–1.5 log more virus than untreated cells.

Discussion. Our findings on the enhancement of HVS and HVA and their antigens suggest that 70–75% nonconfluent cell monolayers and exposure of cells to virus before treatment with TPA resulted in higher titers for both viruses as well as higher yields of EA and LA antigens. The increase in virus titers is significant since these viruses do not yield high titers regardless of the cell lines used. Thus the interaction of TPA with HVS and HVA should enable high virus yields for molecular, biological, immunological, and *in vivo* investigations. Interaction of TPA with oncogenic HVS and HVA in semipermissive cells such as human and CS skin cells may influence the transformation of such cells. The increase in number of plaques in cells infected with virus and treated with TPA shows that the number of plaques did not fully correlate with the infectious virus titers in either the permissive or semipermissive cells. It is possible that TPA may be influencing an increase in incomplete virus and/or those viral proteins which may be responsible for CPE.

Present investigations on enhancement of EBV-VCA and EBV-EA confirm and extend the findings of Zur Hausen *et al.* (8). The percentage enhancement of EA and VCA from B95-8 and P_3HRl cells were higher in their studies (55 and 65%) than reported here (15 and 28%). The difference may be due to impurities in the TPA, rapid oxidation of TPA, or perhaps due to different batches of cells. We have found that different batches of TPA gave variable results in enhancement of EBV virus antigens, mitogenic response (3), and production of infectious HVS and HVA. Our data also show that under the same experimental conditions both human and nonhuman primate cells responded differently to individual TPA doses in achieving maximum enhancement or induction of antigens (Fig. 3). Similar to our data Zur Hausen *et al.* (8) showed that more antigens were produced in P_3HRl and B95-8 cells than other animal and human cells using a single dose of TPA. Fig. 3 also suggests that TPA can influence the regulation of expression of EBV genome in cell lines which have undergone many subcultures and has limited effect in those cell lines which have been recently established. To our knowledge B95-8 and P_3HRl cell lines are over 200 passages whereas AG, Landis, 407-I, and 731-C are less than 20 passages. Lin *et al.* (17) were able to obtain approximately 18-fold higher yields of purified EBV-DNA from P_3HRl cells after TPA treatment, and superinfection of Raji cells with TPA stimulated P_3HRl virus showed about a five-fold increase in recovery of label incorporated into EBV-DNA. Our preliminary data suggest that cell concentration and cell viability could influence cell–TPA interactions. The lower virus yields in TPA-treated producer cells could be due to production of more noninfectious virus particles. Thus the combination of high cell concentration (2×10^6) and low viability (<60%) could result in a poor response to TPA, indicating that cells must be in log phase in order to interact with TPA.

Although there have been reports of the promoter effects of TPA in various cell transformation systems (1, 2), the mechanism of action is not known. A study of the interactions of TPA, carcinogens (including carcinogenic viruses), and tumor cells would help delineate the roles of tumor promoters, carcinogens, and tumorigenic viruses in the induction of neoplasia. Since TPA enhances cell transformation by transforming viruses (3, 9, 10), it may also prove effective in enhancing tumor production in animal models. In the case of nasopharyngeal carcinoma, for instance, it is postulated that the disease may be caused by the combined effects of carcinogens with EBV (18, 19).

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