RESEARCH REPORT

Effect of honey bee venom on differentiation of cholinergic neurons

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ABSTRACT

Mouse P19 embryonic carcinoma (EC) cells are pluripotent and can differentiate into a population consisting largely of neurons and glia cells using a concentration of $5x10^{-7}$M of retinoic acid (RA). Thus, P19 EC cells are a good model system to study events occurring during the critical phases of neuronal differentiation, *in vitro*. Honey bee venom (BV) consists of mellitin, phospholipase A2, apamin and several other bioactive substances. Previous studies have shown that mellitin and phospholipase A2 – two major components of BV – play an important role in the differentiation of neurons. The purpose of this study was to examine effects of BV and RA on the differentiation of cholinergic neuron in P19 cell line. Preliminary results obtained from morphological examination showed that six days after treatment with $5x10^{-7}$M RA, P19 cells produced processes, and gradually obtained neuronal phenotype at approximately day-10. All cells then died at day-11. P19 cells treated with 1.3µg/ml BV produced processes on day-6 and neurons appeared in the next four days. They then proceeded to total size until day-10 and produced elongated processes; however, all cells died on day-11. Using BV and RA together had the same effect but more pronounced differentiating results. It can be concluded that applying BV with RA has an additive effect on cell differentiation and proliferation. The presence of acetylcholinesterase (AChE), frequently used as a marker for neuronal differentiation, was also determined and found using DTNB.

KEYWORDS: P19 cells, neuron, differentiation, honey bee venom, retinoic acid, Acetylcholinesterase (AChE)

INTRODUCTION

P19 embryonic carcinoma cells are a murine cell line capable of differentiating into a wide variety of cell types when aggregated and grown in the presence of dimethyl sulfoxide (DMSO). P19 cells can differentiate into cells of mesodermal and endodermal origin, such as cardiac and skeletal muscle and epithelium (Pachernik et al, 2004). When treated with retinoic acid (RA) they can differentiate into a different spectrum of cell types, including neurons and astroglia – the cell types normally derived from embryonic neuroectoderm. P19 Cells expressing neuronal markers appear within 4-5 days after RA treatment *in vitro*, while cells expressing astrocyte markers do not appear until days 9-10. Nevertheless, similar doses of RA and other drugs are required to induce the development of these two cell types, suggesting that they may develop from a common progenitor cell (Resende et al, 2007; Soprano et al, 2007; Resende et al, 2008).

Retinoic acid (RA), the derivative of retinol, and its signaling pathways, which involve retinoic acid (RAR) and retinoid X (RXR) nuclear receptor-families, play significant roles in the regulation of cell proliferation, differentiation, and apoptosis (Arioka et al, 2005; So et al, 2006, Ziouzenkova and Plutzky, 2008). *In vitro*, RA induces the pluripotent embryonic carcinoma (EC) cells to differentiate into various lineages, depending on both the concentration of RA and cell culture conditions (Yao et al, 1995; Bastien and Rochette, 2004).

The composition of honeybee venom (BV) consists of melittin, phospholipase A2, apamin, mast cell degranulating peptide and several bioactive amines, such as histamine

and epinephrine (Peiren et al, 2005). Melittin and phospholipase A2 are two major components of BV, in quantities of about 40-60% and 15-20%, respectively, which are generally thought to play an important role in the induction of the irritation and allergic reaction associated with the bee stings (Kwon et al, 2004; Yue and Kumamoto, 2005).

MATERIALS AND METHODS

Bee venom

The Iranian Honey Bee (*Apis mellifera*) venom was prepared by placing bees on a 6mm wire grid, which was electrically pulsed. The bees then produced venom that dropped onto a glass slide, which was collected from the glass and freeze-dried according to the method of Lariviere (Lariviere and Melzack, 1996).

Cell culture

EC P19 cells were obtained from Iran Pasteur Institute, Tehran, and were cultured in alpha minimal essential medium (α-MEM) (GIBCO,USA) supplemented with 2.5% (v/v) fetal calf serum (GIBCO,USA), 7.5% (v/v) calf serum (GIBCO,USA), and 100µg/ml each of penicillin and streptomycin. Cells were routinely sub-cultured every 2 days by treating them with Ca^{+2} and Mg^{+2}-free phosphate-buffered saline (PBS) containing 0.025% (v/v) trypsin and 1mM EDTA and dispersing into fresh medium, and were maintained at 37°C in a 5% (v/v) CO_2.

Cell proliferation assay

P19 cells were cultured on 96-well plates ($3x10^4$ cells/well) in α-MEM containing 2.5% (v/v) FBS and 7.5% (v/v) calf serum for 24hr, then treated with or without RA ($5x10^{-7}$M) and BV at concentrations of 1, 3, 5, 7, 9, 11µg/ml for 2-5 days; the culturing media were refreshed every 2-days. MTT assays were performed on days 1, 3 and 5. At a fixed time on each day, 10µl MTT (5mg/ml) was added to each well containing 100µl culture media and was carefully mixed. The mixtures were incubated at 37°C for 4hr, and 100µl isopropyl alcohol (containing 0.04mM HCl), was added and incubated for 5hr. The optical absorbance was determined at 570nm with an ELISA reader.

Differentiation of P19 cells with RA ($5x10^{-7}$M)

Cell differentiation was carried out as follows: Cells in the exponential growth phase were treated with Ca^{+2} and Mg^{+2}-free phosphate-buffered saline (PBS) containing 0.025% (v/v) trypsin and 1mM EDTA to remove them from the surface of the tissue culture dish. They were plated at a concentration of $3x10^4$ cells/ml into a bacteriological-grade petri dish, where they aggregated spontaneously. The medium containing RA ($5x10^{-7}$M) was replaced after 3 days, and 2 days post-RA treatment the aggregates were plated into tissue culture dishes. Aggregates were scored for the presence of neurons on days 6, 8 and 10 of the culture.

Differentiation of P19 cells with BV (1.3µg/ml)

Cells were plated at a concentration of $3x10^4$ cells/ml into a bacteriological-grade petri dish, where they aggregated spontaneously. The medium containing BV with concentration of 1.3µg/ml was replaced after 3 days, and 2 days later the aggregates were plated into tissue culture dishes. Aggregates were scored for the presence of neurons on days 6, 8 and 10 of culture.

Differentiation of P19 cells with BV (3µg/ml) and RA ($5x10^{-7}$M)

Cells were plated at a concentration of $3x10^4$ cells/ml into a bacteriological-grade petri dish, where they aggregated spontaneously. The medium containing BV (3µg/ml) and RA ($5x10^{-7}$M) was replaced after 3 days, and 2 days after this treatment the aggregates were plated into tissue culture dishes. Aggregates were scored for the presence of neurons at 6 to 10 days period.

Differentiation and quantitative morphology

Morphometric analysis was performed by Ziss inverted microscope linked to a black and white Nikon camera. Images of five fields per well were taken with an average of 10 cells per field. The number of differentiated cells was determined by microscopic examination of the field and counting cells that had at least one neurite with a length equal to the cell body diameter, and expressed as a percentage of the total cells in the field. Neurite growth was determined by manually tracing the length of the longest neurite per cell (using Image J software) for all cells in a field that had an identifiable neurite and for which the entire neurite arbor could be visualized. Data from five fields in each well were pooled, and each well was designated as an 'n' of one. Experiments were repeated at least three times using cultures prepared on separate days.

Enzyme assay

These assays were carried out using the spectrophotometric procedure described by Ellman et al (1961). The activity specifically attributable to acetylcholinesterase was determined using DTNB (dithionitrobenzoic, Sigma, USA). The cells were removed from the tissue culture dishes by the addition of PBS containing trypsin and EDTA, as above.

Table 1. Effect of RA and different concentrations of BV on P19 cell viability after 1, 3 and 5 days treatment as compared with the control using MTT assay. (means ± SEM) (n = 3 at each group).

Cell viability (% of live cell)			
Experimental group	**Day 1**	**Day 3**	**Day 5**
Control	100 ± 0.00	100 ± 0.00	100 ± 0.00
RA	97 ± 2.00	88.5 ± 3.50	83.5 ± 3.50
BV1	98.5 ± 1.50	78.5 ± 1.50	69 ± 3.00
BV3	95.5 ± 1.50	81 ± 2.00	74.5 ± 1.50
BV5	77 ± 3.00	59.5 ± 3.50	44 ± 2.00
BV7	71 ± 1.00	51.5 ± 2.50	30 ± 2.00
BV9	57 ± 3.00	45.5 ± 2.50	12 ± 2.00
BV11	42 ± 2.00	28 ± 2.00	0 ± 0.00

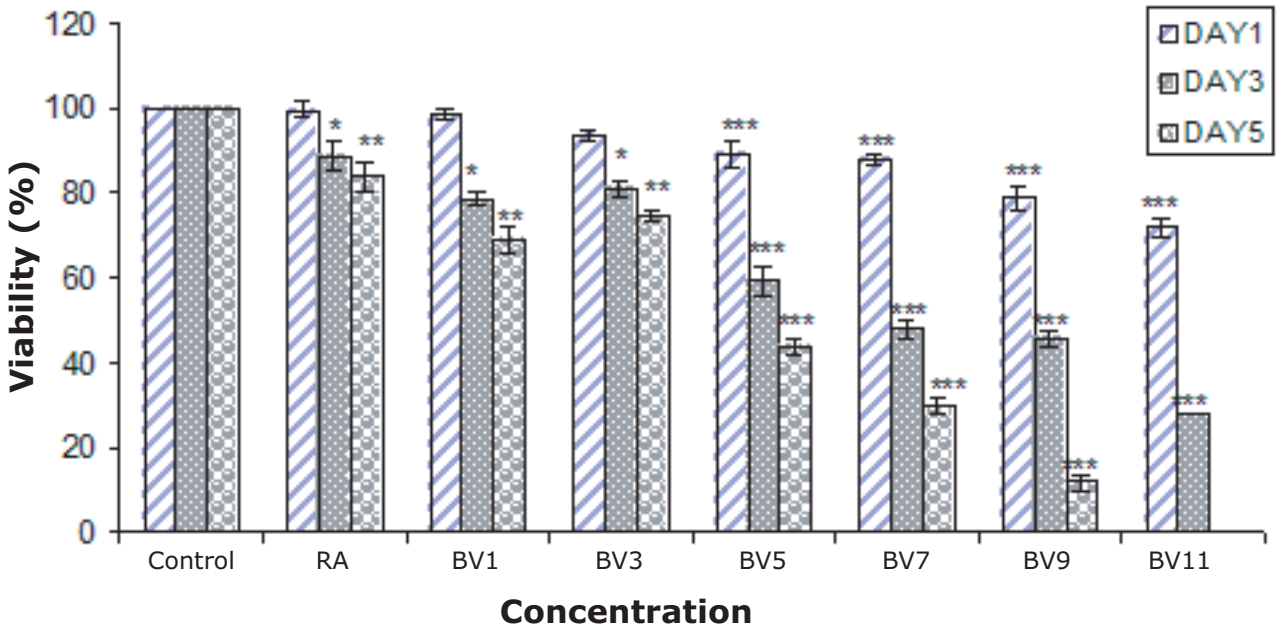

Figure 1. Histograms of proliferative response of P19 cell line to RA ($5x10^{-7}$M) and BV at different doses (1-11µg/ml) after 1, 3 and 5 days treatment as compared to control using MTT assay (means ± SEM) (n = 3 at each group) (*p < 0.05, **p < 0.01, ***p < 0.001).

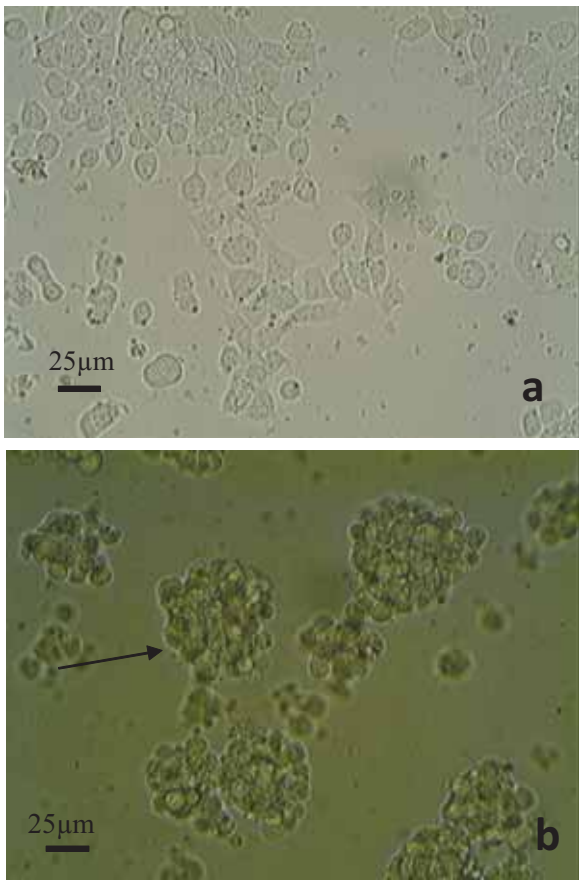

Figure 2. Inverted photomicrographs of undifferentiated P19 cells. (a) P19 cells before aggregation. (b) P19 cells after aggregation on day-6 of culture. Arrows in b show aggregations (magnification 400x).

After sonication, the lysates were stored at -70°C until used in the assay. Protein concentrations were determined according to a modified Rieger et al (1980) procedure (Ellman et al, 1961; Peiren et al, 2005).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made by the analysis of variance (ANOVA) and when significant differences were observed, Tukey's test was employed for multiple comparisons. Stastistical significance was inferred at $P < 0.05$.

RESULTS

Effect of RA and BV on cell viability with MTT assay

In this analysis a concentration of 5µg/ml BV, which caused death of about 50% of total cells, was determined as LD50, and 2 concentrations below LD50 (1.3µg/ml) were used in experiments (Table 1, Figure 1).

Differentiation of P19 cells in monolayer culture with retinoic acid and bee venom (1.3µg/ml)

Pluripotent embryonic carcinoma cells may be induced to differentiate if they are aggregated and cultured in suspension for several days before plating onto tissue culture grade plastic surfaces. For all of our experiments, the aggregates were cultured for 5 days in suspension. They were then plated and examined 2-3 days later when differentiated cells had migrated out of the aggregates. Virtually all RA- and BV-treated aggregates contained some cells with neuronal morphology. When aggregates of P19 cells were plated into tissue culture dishes in the absence of RA or BV, we observed only undifferentiated cells surrounded by a small

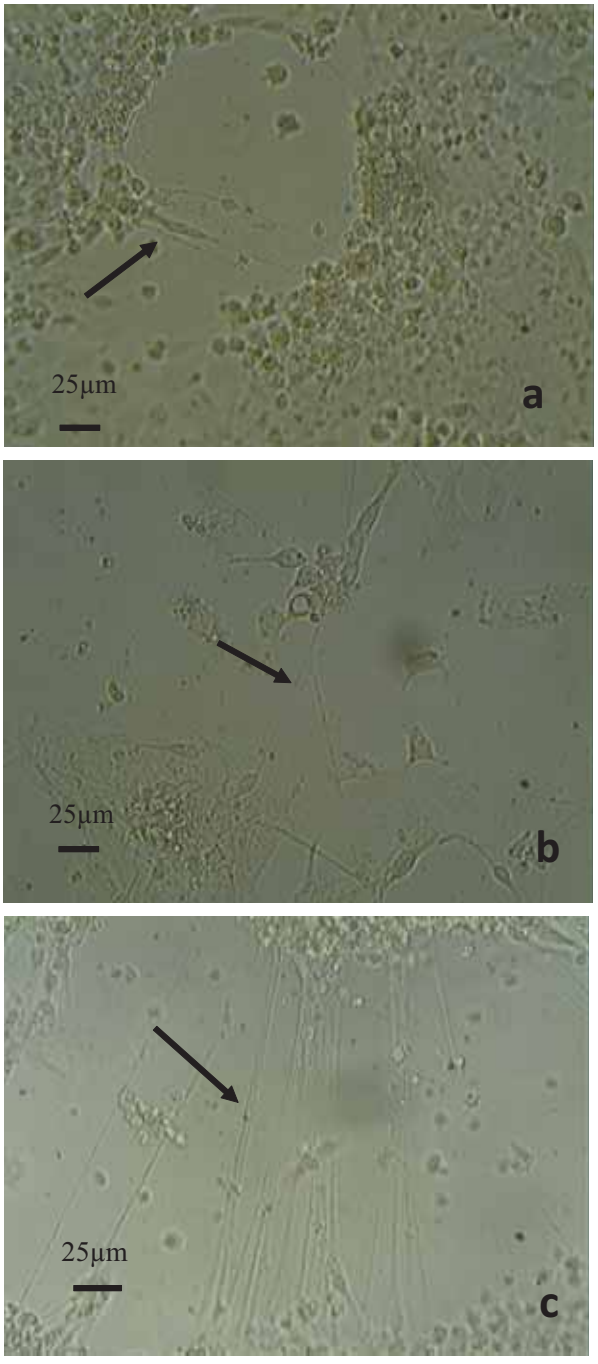

Figure 3. Inverted photo micrographs of P19 cells following 5days treatment with RA ($5x10^{-7}$M). (a) P19 cells on day-6. (b) P19 cells on day-8. (c) P19 cells on day-10. Arrows in a, b and c show processes of differentiated cells (magnification 400x).

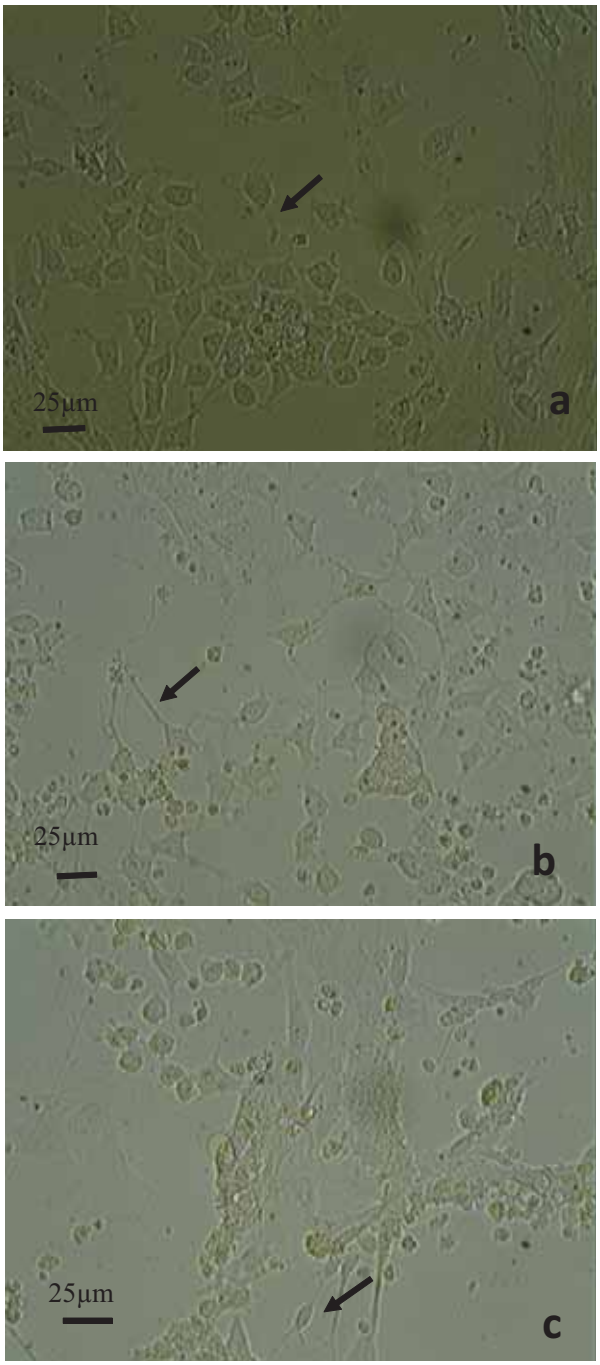

Figure 4. Inverted photo micrographs of P19 cells following 5 days treatment with BV (1µg/ml). (a) P19 cells on day-6. (b) P19 cells on day-8. (c) P19 cells on day-10. Arrows in a, b and c show processes of differentiated cells (magnification 400x).

amount of tissue resembling extra-embryonic endoderm (Figure 2; a and b). When P19 cells were cultured as aggregates in the presence of RA or BV (1.3µg/ml) or RA or BV (3µg/ml) , the cell types which were present 2 days after plating were markedly different. Between 24 and 48 hr after plating, neuron-like cells appeared whose processes grew rapidly from the aggregates. (Figures 3, 4, 5 and 6; a, b and c). In addition, using 3µg/ml BV had higher differentiating effect compared to a dose of 1µg/ml of BV applied, and percentage of differentiating cells were higher when RA and BV (3µg/ml) were used together (Figure 7 and Table 2).

Inverted photomicrographs of these cell types are shown in Figures 2–6. The processes from these neuron-like cells were frequently arranged in bundles. They had multiple branches with tips located on the cell surface.

AChE assay

Acetylcholinesterase (AChE) has been frequently used as a marker for neuronal differentiation although this enzyme is also present in some non-neural tissues (Rieger et al, 1980; Parnas and Linial, 1995). Figure 8

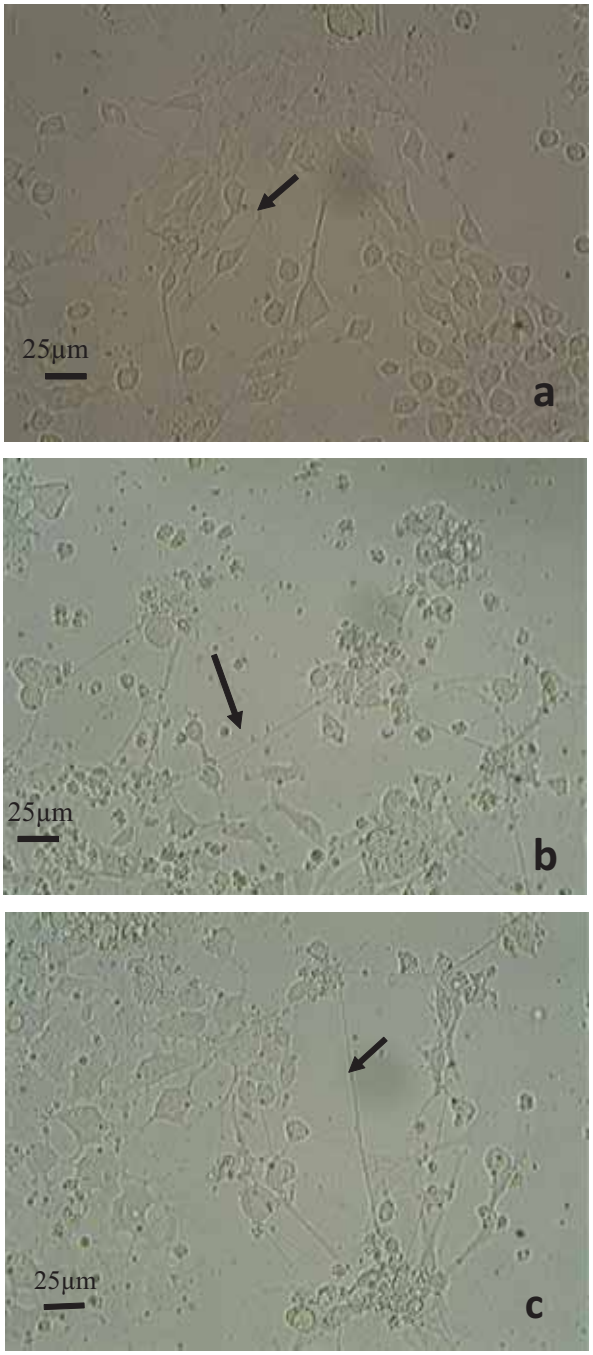

Figure 5. Inverted photo micrographs of P19 cells following 5days treatment with BV (3µg/ml). (a) P19 cells on day-6. (b) P19 cells on day-8. (c) P19 cells on day-10. Arrows in a, b and c show processes of differentiated cells (magnification 400x).

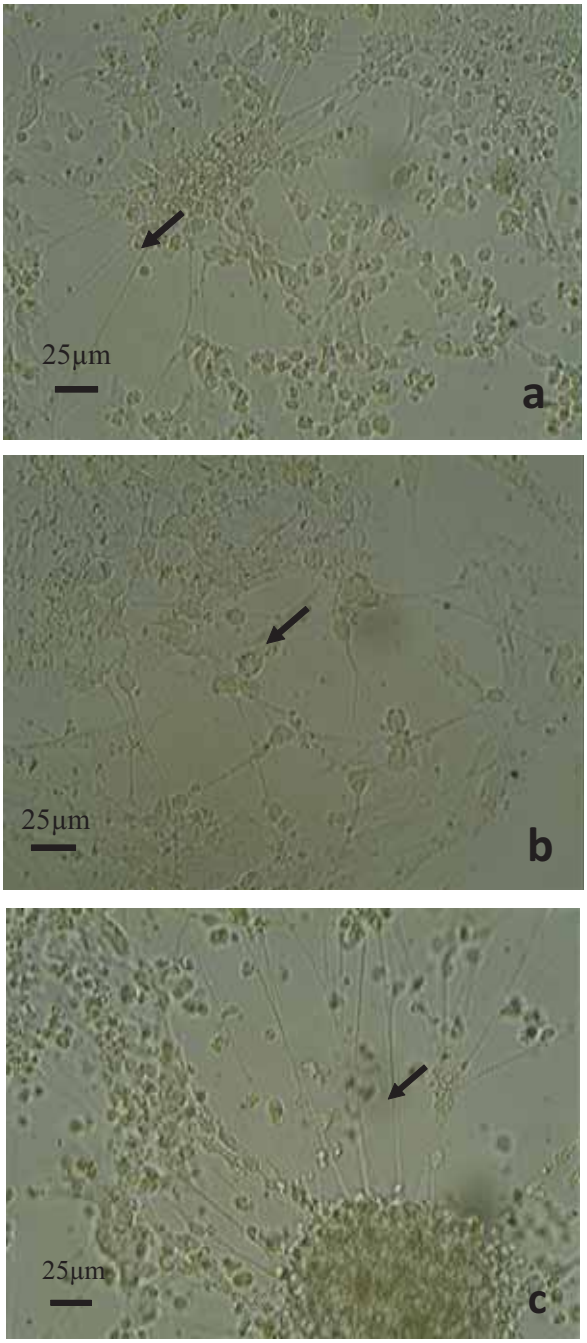

Figure 6. Inverted photo micrographs of P19 cells following 5 days treatment with RA plus BV (1µg/ml). (a) P19 cells on day-6. (b) P19 cells on day-8. (c) P19 cells on day-10. Arrows in a, b and c show processes of differentiated cells (magnification 400x).

and Table 3 shows the result of experiments in which the acetylcholinesterase activity was measured in aggregated cultures of both treated and untreated cells on day-10. Although the absolute values of acetylcholinesterase varied from one experiment to another, the patterns were consistent (see Figure 4 and Table 3 showing representative results). Untreated cultures contained little activity. However, in RA-treated cultures, acetylcholinesterase activity was higher than BV (3µg/ml)-treated cultures and RA and BV (3µg/ml)-treated cultures had the highest activity of all.

DISCUSSION

In this study, P19 cells were used to study the effect of BV, as they are able to differentiate into neurons, *in vitro*. We used RA ($5x10^{-7}$M), BV (1.3µg/ml), and RA plus BV (3µg/ml) to induce differentiation. Surveys for neuronal differentiation were performed using morphological and AChE assay. Our results clearly demonstrated that RA and BV induce neuronal differentiation of P19 cells, and that the rate of differentiation increased when BV and RA were used together in the cell culture media.

Table 2. Effect of 5-day treatment with RA, BV and RA plus BV on neuronal differentiation of P19 cells as compared with the control surveyed on days 6, 8 and 10 of the culture (mean ± SEM) (n = 3 at each group).

Differentiation (%)			
Experimental groups	**Day 6**	**Day 8**	**Day 10**
Control	1.5 ± 0.50	1.75 ± 0.25	2 ± 0.00
RA	66.5 ± 2.50	76.5 ± 6.50	86 ± 4.00
BV1	10 ± 3.00	16.5 ± 1.50	20 ± 2.00
BV3	12.5 ± 2.50	19 ± 2.00	24.5 ± 2.50
RA+BV3	70.5 ± 2.50	79.5 ± 5.50	93.5 ± 3.50

The doses of RA used in the study were not toxic to the P19 cells, and the population of embryonic carcinoma cells was found to be homogeneous with respect to its response to RA treatment. It could be argued that the P19 population as a whole is committed to making neurons, glial, and fibroblast-like cells and that retinoic acid acts to enhance the differentiation process but has no role in the determination of these cells. However, since P19 cells can differentiate into extra-embryonic, endoderm-like cells and into muscle under certain conditions (McBurney and Jones 1982; McBurney and Reuhl, 1988; McBurney, 1993). Villeneuve et al has previously reported that RA at a concentration of $5x10^{-7}$M could induce neuronal differentiation in P19 cells and the highest differentiation percentage they observed was 95% (Villeneuve et al, 1982). In our study the highest differentiation percentage obtained on day 10 was approximately 80% with RA ($5x10^{-7}$M), 20% with BV (1µg/ml), 24.5% with BV (3µg/ml), and 93.5% with RA ($5x10^{-7}$M) plus BV (3µg/ml). Thus our finding show that differentiation percentage with BV was low, but using both BV and RA increased the differentiation potency of RA to 93.5%. Nakashima et al have suggested that PLA2, the most abundant component of BV that has a role in cell differentiation, induced neurite outgrowth in PC12 cells. PLA2 liberates fatty acids from live PC12 cells, and the measuring of these acids is a method to assess phospholipase activity and they showed that inducing neurite outgrowth in PC12 cells was dependent on this activity (Nakashima et al, 2003; Nakashima et al, 2004). Therefore, in this study we used whole BV and our results showed that BV exerts differentiating effects on P19 cells. RA induces release of arachidonic acid and its metabolites which may play an important role in cell proliferation, differentiation, and apoptosis (Heyden and Defize, 2003; So et al, 2006). In brain tissue, arachidonic acid is mainly released by the action of phospholipase A2 (PLA2) and phospholipase C (PLC)/diacylglycerol lipase pathways. Arachidonic acid and its metabolites markedly affect the neurite outgrowth and neurotransmitter release in the cells of neuronal and glial origin (Arioka et al, 2005; Ikeno et al, 2005). We propose that RA receptors coupled with phospholipases A2, C and D in the nuclear membrane play an important role in the redistribution of arachidonic acid in neuronal and non-nuclear neuronal membranes during differentiation and growth suppression (Lariviere et al, 1996). The neurons were identified by their morphology, isoenzyme pattern, and Na^+ channels. Identification of neurotransmitters and associated enzymes is another way of characterizing neurons. This approach was taken by Pfeiffer et al (Pfeiffer et al, 1981), who have isolated a line of embryonal carcinoma cells which appeared to be spontaneously committed to the formation of cholinergic neurons. Elevated levels of acetylcholinesterase were present in differentiated cultures derived from these cells. Thus, we reconfirmed observed-microscopically morphological differentiation of PC12 cells into neurons using AChE assay for each of RA, BV3, and RA+BV3 treatments. This assay confirmed the presence of neuronal cells in our experimental cell cultures.

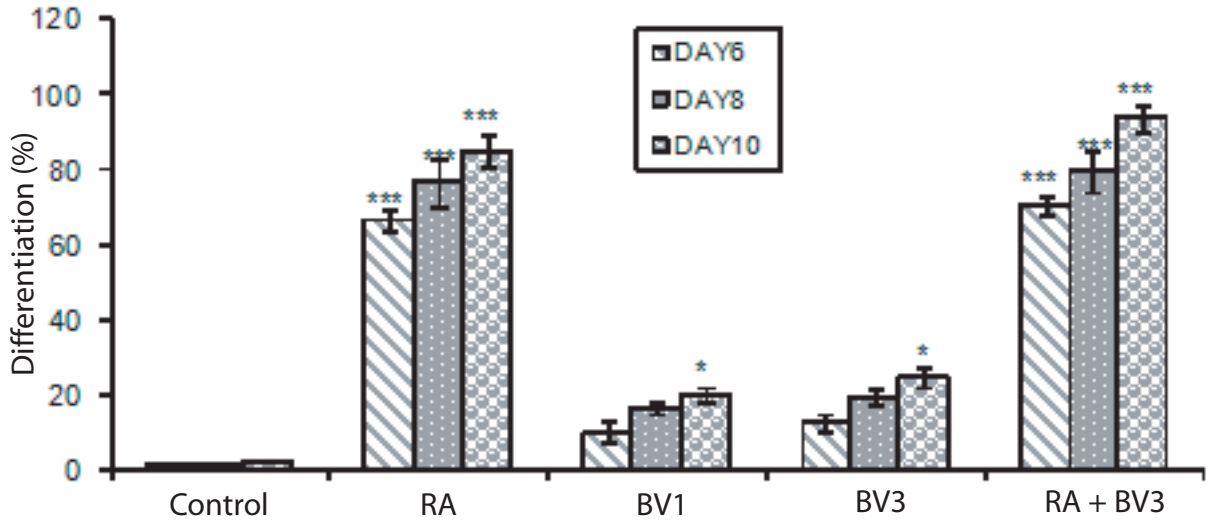

Figure 7. Histograms showing the effects of RA ($5x10^{-7}$M), BV(1.3µg/ml) and RA plus BV treatment on neuronal differentiation of P19 cells as compared with the control group in 6, 8 and 10 days of the culture. (mean ± SEM) (n = 3 at each group) (*P < 0.05,***P < 0.001).

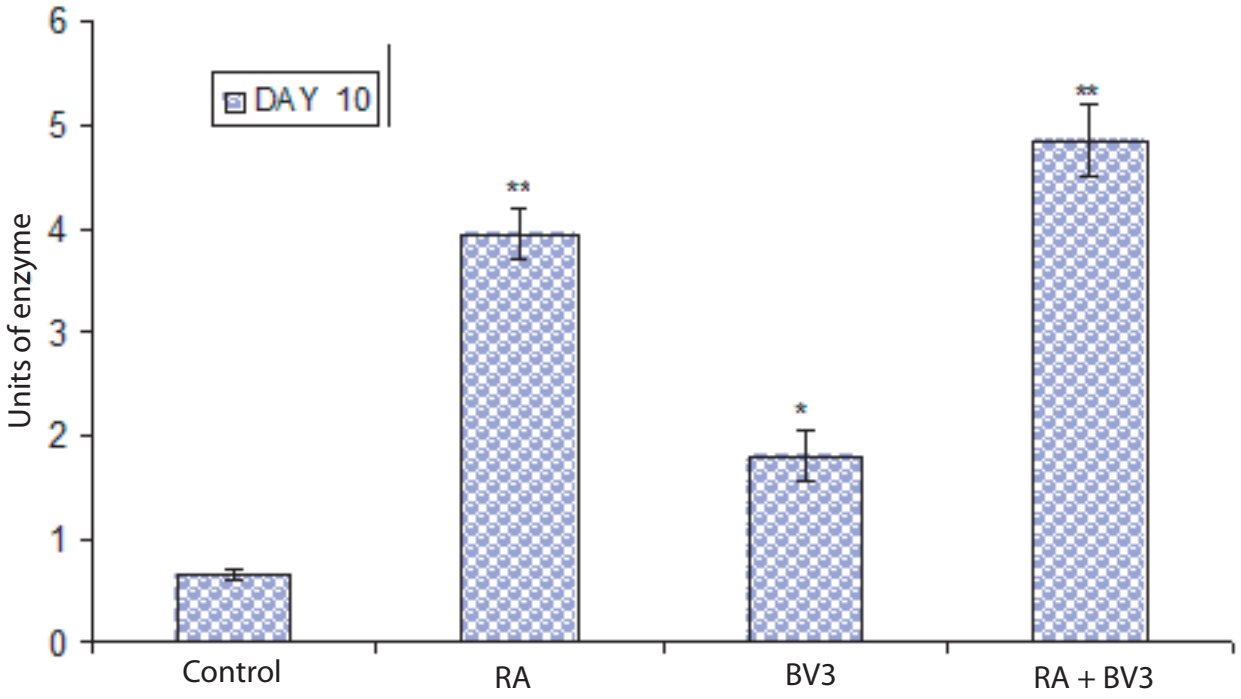

Figure 8. Histograms of acetylcholineterase activity in RA-, BV3-, RA plus BV3-treated P19 cells as compared with the control after 10 days of the culture. (means ± SEM) (n = 3 at each group) (*p < 0.05, **p < 0.01, ***p < 0.001).

Table 3. Comparison of neuronal differentiation marker (acetylcholineterase) activity in RA-, BV3-, RA+BV3-treated P19 cells after 10 days of culture (mean ± SEM) (n = 3 at each group).

Experimental Group	Units of enzyme (Day 10)
Control	1.2 ± 0.05
RA	4 ± 0.25
BV3	1.9 ± 0.25
RA+BV3	5 ± 0.35

CONCLUSIONS

In this study we used whole bee venom and retinoic acid to investigate their effects on differentiation of PC12 cells into neurons. By employing microscopic observations and acetylcholinesterase assays we found that the maximum differentiating effects were displayed when both RA and BV were used together.

ACKNOWLEDGMENT

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