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Effect of *Leiurus quinquestriatus* venom and venom fractions on cells cultured *in vitro*

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ABSTRACT

Most previous studies on scorpion venom have considered in vivo effects on tissues and cells only. The present study assess the effects of venom of the scorpion *Leiurus quinquestriatus* on the growth of three cell lines: baby hamster kidney (BHK-21), African green monkey kidney (Vero) and Buffalo green monkey kidney (BGM). Crude venom (at 0.03, 0.06, 0.09 and 0.12 µg/ml.) decreased cell growth within 24h which was significant at 0.03 and 0.09 µg/ml on the BHK-21 and Vero cells, respectively indicating cytotoxicity. Five fractions of *L. quinquestriatus* venom, either decreased or increased cell growth. Fraction four significantly increased cell growth of Vero and BGM cells, implying a mitogenic effect.

KEYWORDS: *Leiurus quinquestriatus*, venom, cell lines.

INTRODUCTION

Most studies of scorpion venom are concerned with the toxicological, cardiovascular, respiratory and pharmacological effects (Freira-Maia *et al.* 1973; Eitan *et al.* 1990; Abrough *et al.* 1992; Ismail *et al.* 1994; Moskowitz et al. 1994; Fletcher *et al.* 1996; Cestele *et al.* 1997). Ibrahim (1990) reported that scorpion and snake venom stimulate cell division. Abd El-Rehim (1990) showed that bradykinin potentiating factor (BPF) isolated from the venom of the scorpion *Buthus occitanus* has a mitogenic effect on both duodenal mucosal cells of the mouse (*in vivo*) and baby hamster kidney (BHK-21) cells (in vitro). Salman (1995) showed that BPF induced a faster recovery of burnt guinea pig skin compared to other drugs. However, there are no detailed accounts of the effect of scorpion venom and venom fractions on cell growth. The current study tests the effect of crude scorpion (*L. quinquestriatus*) venom and venom fractions on the growth of BHK-21, BGM and Vero cell lines.

MATERIALS AND METHODS

Venom supply: *Leiurus quinquestriatus* crude venom (200 mg) was obtained by electric stimulation of the telson. Lyophilized venom was reconstituted in 0.9 % NaCl.

Fractionation of the venom: Crude *L. quinquestriatus* venom was fractionated (using a 2.5 x 90 cm column Sephadex G-50 equilibrated with dextran-2000 at room temperature) according to Possani *et al.* (1981). Fractions of the toxin (3.75 ml) were eluted at a flow rate of 90 ml/h. The presence of total protein in each fraction was assessed by spectrophotometery at 280 nm and five protein peaks were identified. Appropriate fractions were pooled, then dialyzed against distilled water, lyophilized and stored at –20°C.

Cell lines: Three cell lines (baby hamster kidney (BHK-21: MacPherson & Stocker 1962), adult African green monkey kidney (Vero: Yasumura & Kawakita 1963) and Buffalo green monkey kidney (BGM: Dahling *et al.* 1974) kindly provided by Virology Department, U.S. Naval Medical Research Unit No.3 (NAMRU-3), Cairo Egypt.

Cell preparation: Cells were trypsinized, suspended in Eagle's minimal essential medium (MEM) (GIBCO, USA) with 5% fetal calf serum. Cell viability was tested using Trypan blue

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exclusion. The cells were evenly distributed in 96-well microtiter plates, with 5000 cells per well, and incubated at 37°C, 5% CO_2. After 24 h, the medium was replaced with MEM/0.5 % serum in order to synchronize cell growth. On the third day either crude venom (0.03, 0.06, 0.09 and 0.12 µg/ml) or venom fractions (1, 2 and 10 µg/ml) in MEM/10 % serum were added to the cells. BHK-21 cells were used to study the effect of the concentrations of crude venom alone on the cell growth after 24 h of incubation. Vero cells were used to study the effect of crude venom and venom fractions on cell growth during 96 h. The initial constant viable cell number (5000) and recounted after 24, 48, 72 and 96 h of incubation with the venom. BGM cells were used to study the effect of the crude venom and venom fractions on the cell growth after only 24 h of incubation with the venom. Ten wells were used in each case as a control and similar number were used for each concentration of the crude venom and each venom fraction.

Statistical analysis: Data were analysed using Analysis of variance with the program Statgraphics.

RESULTS

The effect of crude *L. quinquestriatus* venom on the growth of BHK-21 cells after 24 h of treatment is shown in Figure 1a. At the lower concentrations of 0.03 and 0.06 µg/ml, there was clear decrease in the growth of BHK-21 cells: the concentration of 0.03 µg/ml caused the maximum decrease, by about 31% of the control level (see Fig 1a). Increasing concentrations had gradually decreasing effects of cell growth compared to the control.

The effect of crude *L. quinquestriatus* venom on Vero cells after 24, 48, 72 and 96 h of treatment is shown in Figure 1b. A decrease in the number of cells relative to the control is obvious at all venom concentrations throughout the test period, even at the highest concentration of 0.12 µg/ml. For all incubation times, the maximum decrease recorded for venom-treated cells was obtained at a venom concentration of 0.09 µg/ml, with the lowest value after 96 h of about 50% of the control cell number (Fig. 1b).

On the other hand, crude scorpion venom did not decrease the number of BGM cells, but instead there was a slight but statistically significant increase recorded after 24 h incubation using 0.06 and 0.09 µg/ml (by 9.5% and 8.7% respectively: Fig. 1c).

The results of the experiment on venom fractions (Fig. 2) showed that fraction 1 induced a dose-dependent decrease of Vero cells, reaching a maximum at 10 µg/ml (23% of the corresponding control cell number). Fractions 2, 3 and 4 showed a significant increase of Vero cells (14, 23 and 35.5% at 1, 2 and 10 µg/ml respectively), though fraction 4 was the most effective and 2 was the least effective. Concerning the effect of the same venom fractions on BGM cells, it is clear from Figure 3 that fractions 2 and 3 induced a slight but statistically significant increase in cell numbers at the low concentration used (1 and 2 µg/ml), and that the lowest (1 µg) was more effective. However, fraction 4 applied to the cells recorded the highest mitogenic effects of all concentrations used (about 109%, 119% and 125% for 1, 2 and 10 µg/ml). Fraction 1 had no effect on either cell lines.

The results presented in Figure 2 show that fraction 1 at a concentration of 1 µg/ml enhanced the reduction in Vero cell number after 48 h to reach 15% of the corresponding control level. However, after 72h the reduction of these cells had returned almost to that recorded after 24 h. Doubling the dose of this venom fraction or increasing it to 10 µg/ml enhanced the decrease of cell growth remarkably, reaching 41% and 37% of the corresponding control values respectively after 48 h. This effect increased still further, but only slightly, after 72 h incubation, reaching 46% and 51% respectively.

The increases in the number of Vero cells recorded for all concentrations after 24 h for fraction 3 was abolished after 48 and 72 h, but those recorded for fraction 4 only reduced

slightly after 48 h, and only a bit more by 72 h, but still statistically significant at each concentration level compared with the corresponding control values (Fig. 2).

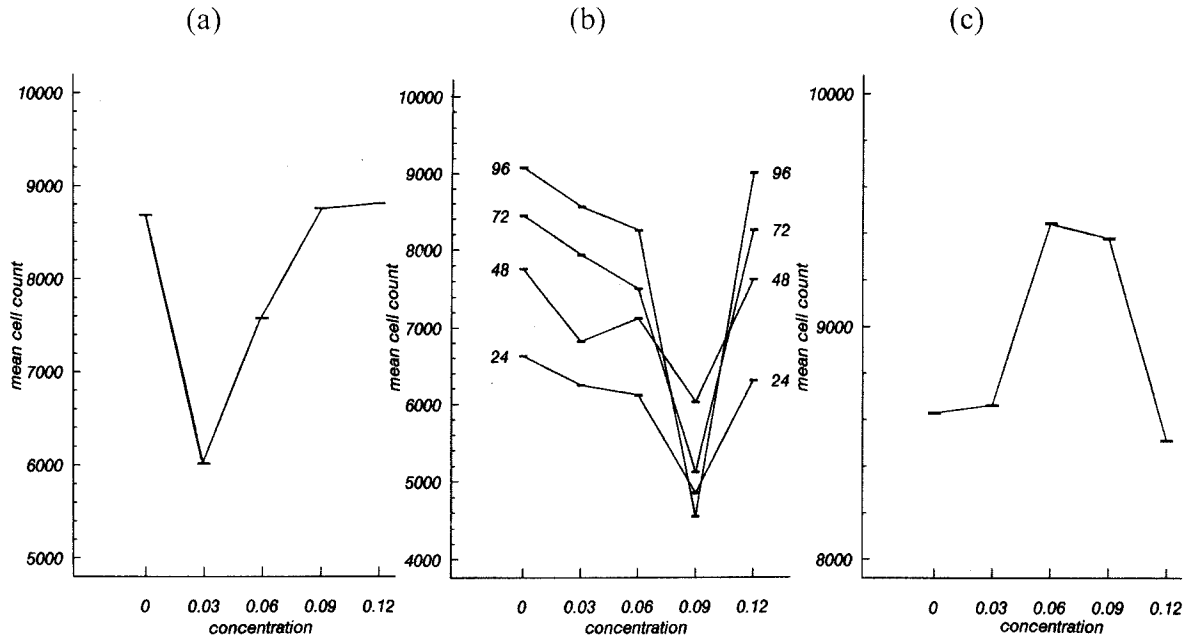

Figure 1: The effect of crude *Leiurus quinquestriatus* venom at different concentrations (µg/ml medium) on:-

a) the growth of BHK-21 cells after 24 h. The treatments have highly significant effects on cell growth ($F_{4,20}$ = 54862, $p<0.001$).
b) the growth of Vero cells after various periods of time. Both treatments have highly significant effects on cell growth (time $F_{3,71}$ = 172216, $p<0.001$; concentration $F_{4,71}$ = 262596, $p<0.001$; interaction $F_{12,71}$ = 27136, $p<0.001$).
c) the growth of BGM cells after 24 h. The treatments have highly significant effects on cell growth ($F_{4,20}$ = 13505, $p<0.001$).

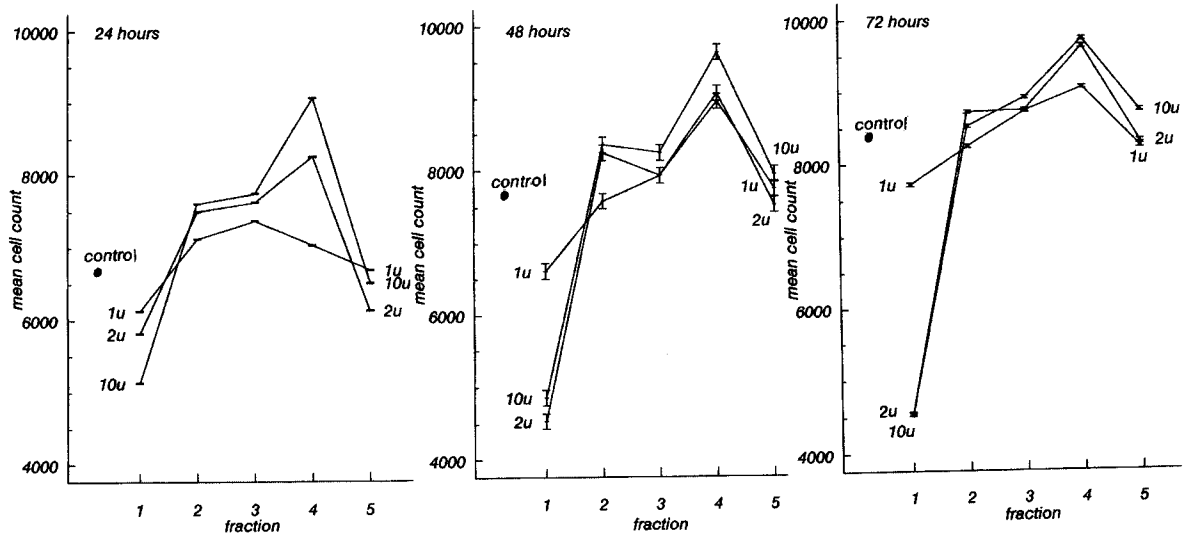

Figure 2: The effect of five *Leiurus quinquestriatus* venom fractions at different concentrations (µg/ml medium) on the growth of Vero cells after various periods of time. All treatments have highly significant effects on cell growth (concentration $F_{2,180}$ = 176, $p<0.001$; fraction $F_{4,180}$ = 14736, $p<0.001$; time $F_{2,180}$ = 4698, $p<0.001$; all interactions are significant, $F > 162$, $p<0.001$).

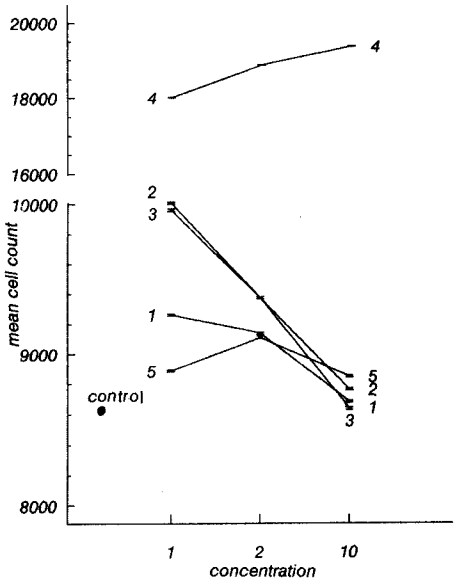

Figure 3: The effect of five *Leiurus quinquestriatus* venom fractions at different concentrations (μg/ml medium) on the growth of BGM cells after 24 h. Both treatments have highly significant effects on cell growth (concentration $F_{4,45} = 1000000$, $p<0.001$; fraction $F_{4,45} = 14000$, $p<0.001$; interaction $F_{8,45} = 12831$, $p<0.001$)

DISCUSSION

Several studies have been concerned with the direct cytopathic and cytotoxic effects of crude venom and venom fractions in vitro (Donta & Shaffer 1980; Iwaguchi *et al.* 1985; Chaim-Matyas & Ovadia 1989; Rhonda *et al.* 1989; Monette *et al.* 1994; Yuhi *et al.* 1996) and *in vivo* (Unkovic-Cvetkovic *et al.* 1983; Damorou *et al.* 1985; Iwaguchi *et al.* 1985; Hofmann *et al.* 1993).

These studies show that the venom binds to calcium ion channels of the cells to act on a specific Ca^{++} channel leading to the activation of protease within the cell (Duncon 1978), formation of pores in the cell membrane (Chang 1974; Harvey *et al.* 1982) and affecting specific molecules of plasma membrane rather than non-specific cell membrane destruction (Son & Walker 1986).

The crude venom of the scorpion *L. quinquestriatus* and its five fractions were tested on the growth of BHK-21, Vero and BGM cell lines and the mean cell number. The present results, however, revealed that the crude venom at the two higher concentrations used (0.09 and 0.12 μg/ml) induced an insignificant decrease of BHK-21 cells. The concentrations 0.03 and 0.06 μg/ml after 24 h induced a significant decrease of the same cells. This decrease was more pronounced at the lower dose. The growth of Vero cells was slightly decreased at concentrations 0.03, 0.06 and 0.12 μg/ml. This decrease, however, was statistically significant only at the two low concentration levels, insignificant at the higher one. At a dose of 0.09 μg/ml the decrease was remarkable. On the other hand, BGM cells treated with the venom showed either undetectable changes at the 0.03 and 0.12 μg/ml concentrations or a slight but statistically significant increase of cell numbers at concentrations of 0.06 and 0.09 μg/ml after 24 h.

These results indicate that the venom contains cytotoxic factors and/or growth inhibitory factor(s) together with mitogenic stimulating factor(s) that may counteract cell growth. Furthermore, there are variable sensitivities of the cells to this venom. Vero cells showed a decrease in growth, mostly in a dose dependent manner, but only if the high concentration values are ignored (0.12 μg/ml, which did not change the cell number significantly). The crude venom at the same concentration levels possessed almost the same pattern of effect during the 96 h test period. The results depict a sensitivity of Vero cells to *L. quinquestriatus* crude venom, with the most effective dose being 0.09 μg/ml. A myotoxic effect of scorpion venom on the myocardium cells was reported by Gueron & Sofer (1991); Sofer *et al.* (1991); Abrough *et al.* (1992); Gueron *et al.* (1992). Several toxins affect the cytoskeletal components of the cell (Reunes *et al.* 1987; Fiorentini *et al.* 1988) subsequent to inhibition of cytoplasmic division.

Concerning the venom fractions of *L. quinquestriatus*, the results indicate that while fraction 1 induced a significant decrease of Vero cell number in a concentration dependent manner, there was insignificant change in the number of BGM cells after 24 h. These

variations in the effects of fraction 1, i.e. a decrease of Vero cells and no change or even slight increases of BGM, might be an indication of variation in the sensitivity of the two cell lines to the action of this fraction. *L. quinquestriatus* venom is known to posses a direct cytotoxic action on different tissues (Moustafa *et al.* 1974; El-Asmar *et al.* 1979a; Gueron *et al.* 1980, 1993; Sofer & Gueron 1988; Sofer *et al.* 1991).

Fractions 2 and 3 induced an appreciable growth increase of Vero cells after 24 h of incubation with comparable efficiency, though the effect of fraction 3 on cell growth was slightly greater than fraction 2. Both fractions, however, induced a comparable increase of BGM cell growth also, yet this increase was lower with increasing dose. In reality, the high 10 µg/ml dose of fraction 3 was almost without effect on BGM cells. This might be due to an increased cytotoxic effect of the fraction components. Thus the factor(s) leading to cell mitosis is not constantly stimulating the events of the cycle leading to cell division. The increase of cell number induced by fractions 2 and 3 might be correlated with opening of special Ca^{++} channels. Ciapa *et al.* (1994) reported that transient changes in intracellular calcium punctuate the cell cycle in various types of cells in culture and in early embryos.

On the other hand, fraction 4 of *L. quinquestriatus* venom induced a dose-dependent increase of both Vero and BGM cells after 24 h of incubation. This increase was more pronounced in the case of BGM cells than Vero cells (108, 119 and 125% in case of BGM compared with 14, 23 and 36% for Vero cells after application of 1, 2 and 10 µg/ml respectively). The last result is interesting as the BGM-cell response was remarkably greater than that of Vero cells. This may be due to the fact that fraction 4 is more or less pure, forming a single electrophoretic band with a molecular weight of about 97,324 (El-Ghitany 1998), which means that it is not a small peptide, even though it has mitogenic activity.

The presence of phospholipase A2 (PLA_2) in scorpion venoms has been reported by Venkalah (1989); Ismail *et al.* (1992) and Gowda & Middlebrook (1993). PLA_2 from the venoms of snakes and scorpions has common characteristics (Venkalah 1989) associated with several toxic, pathological or physiological processes including cellular proliferation (Lambeou *et al.* 1994). The role of PLA_2 in producing kinins is well documented (Lahiri & Chaudhuri 1983; El-Asmar 1984; Ismail *et al.* 1992; Muthalif *et al.* 1996; Xing & Insel 1996). According to Teixeira *et al.* (1994) venom phospholipases can trigger the release of arachidonic acid and lysophospholipids from the membranes. Lysophospholipids can be further transformed into biologically active platelet-activating factor (PAF). Free arachidonate is metabolized by the cyclo-oxygenase enzyme system to form eicosanoids (prostaglandin, thromboxones and leukotrienes), while other venom components can activate endogenous phospholipases which result in the release of eicosanoids and PAF (Teixeira *et al.* 1994). Furthermore, norepinephrine promotes extracellular Ca^{++} influx leading to increase Ca^{++}-calmodulin dependent protein kinase II activity, which in turn activates mitogenic actiprotein kinase leading to the activation of cytoplasmic PLA_2 that releases arachidonic acid (Muthalif *et al.* 1996).

Cattaneo *et al.* (1993) reported that the extracellular calcium plays a stimulatory role in the proliferation of the GLC8 cell line. L-type calcium blockers at concentrations higher than those required to block L-type channel function inhibit (^3H) thymidine incorporation in these cells, which may explain the decrease of cell growth at certain concentrations of high venom fractions. However, calcium-channel antagonists inhibit the growth of murine Swiss 3T3 fibroblasts which do not possess L-type Ca^{++} channels.

The presence of catecholamines in scorpion venom and their release, especially norepinephrine by *L. quinquestriatus* venom, was reported by El-Asmar (1984). Furthermore, a direct PLA_2 action on the proliferation of Swiss 3T3 fibroblasts has been reported to be via specific binding sites. Pancreatic type of PLA_2 in active form specifically recognizes the sites and stimulates thymidine incorporation in DNA (Arita *et al.* 1991). Moreover, the release of

kinins by scorpion venom has been reported by El-Asmar (1984), Ismail *et al.* (1974 a,b; 1992). Ismail *et al.* (1992) reported that aprotinin, a kallikezein-kinin inhibitor that protects both rats and rabbits against *L. quinquestriatus* venom, induces pulmonary oedema. These kinins are either formed through kinin-releasing enzymes or potentiated by a factor present in the snake and scorpion venoms (Nassar *et al.* 1989, 1990; Bailey *et al.* 1991; Yabuki *et al.* 1991; Ismail *et al.* 1992; Ferreira *et al.* 1992, 1993; Antunes *et al.* 1993). Hassanein (1988) and Nassar *et al.* (1989) reported the isolation a bradykinin-potentiating factor from the venoms of the scorpion's *B. occitanus, L. quinquestriatus* and the snake *Naja haje haje.* This fraction obtained from *B. occitanus* venom increased the number of primary multilaminar follicles, secondary follicles and the number of Graafian follicles of premature mice. The number and size of the uterine glands and the endometrium increased also (Nassar *et al.* 1990). *In vivo*, the fraction induced a remarkable increase of the mitotic index of the duodenal mucosal cells, while *in vitro* it almost doubled the number of BHK-21 cells within 24 h as compared with the control. The authors related this to the enhancement of the bioactivity of endogenous bradykinin in vivo and in vitro (Abd El-Rehim 1990). Furthermore, it enhanced the repair of skin burning of guinea pigs even more rapidly than the standard burning drugs (Salman 1995).

Osugi *et al.* (1987) reported that bradykinin increases intracellular Ca^{++} level and the formation of inositol mono-, di- and triphosphates in neuroblastoma x glioma hybrid (NG 108-15) cells. It induces the synthesize of PLA_2 in murine fibroblasts and endothelial cells (Becherer *et al.*, 1982 ; Claesson 1980; Hassid 1983), regenerates burnt skin of guinea pig (Salman 1995) and leads to the increase of cellular cAMP levels (Grenier *et al.* 1982) in cultivated renal epithelial cells.

In conclusion, the increased growth of the cells due to the application of *scorpion L. quinquestriatus* venom fractions might be through one or more of the following ways. The first is the increased influx of calcium ions to the cells that stimulates their proliferation. The second is through PLA_2 activity of these fractions that leads directly to the stimulation of thymidine incorporation in DNA synthesis, and the release of arachidonic acid which is then transformed to prostaglandins, especially PGE_2 and interleukins (IL_2 and IL_3) that stimulate cell growth. The third possible way of stimulating cell division may be through the endogenous release of kinins and their activation. The release of kinins could be through kininogenase or protease action of the venom fraction. To know any of these pathways definitely needs further investigation.

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الملخص العربي

تأثير سم العقرب "*لوريس كوينكويستریاتس*" ومكوناته على الخلايا المزروعة

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أوضحت بعض الدراسات السابقة أن سموم بعض أنواع العقارب أو مكوناتها لها تأثيرات مختلفة على نمو الخلايا العادية أو الخلايا والأورام السرطانية، فبعض هذه السموم يؤدي إلى تثبيط نمو هذه الخلايا والبعض الآخر يعمل على تنشيط هذا النمو. وتهدف هذه الدراسة إلى إلقاء الضوء على تأثير سم أحد العقارب الموجودة في مصر "*لوريس كوينكويستریاتس*" من خلال اختبار كل من السم الخام لهذا العقرب وخمس من مكوناته – عند تركيزات أقل من الجرعة المميتة– على نمو ثلاثة أنواع من الخلايا المزروعة وهي Vero و BHK-21 و BGM. وقد أوضحت النتائج أن معالجة الخلايا بالسم الخام بالجرعات ٠,٠٣ ، ٠,٠٦، ٠,٠٩ & ٠,١٢ ميكروجرام/ سم٣ من الوسط المغذي للخلايا كان لها استجابات مختلفة حسب كل نوع من أنواع الخلايا بعد ٢٤ ساعة من المعالجة. فقد وجد أن الجرعة ٠,٠٩ ميكروجرام /سم٣ تسبب أعلى تأثير مثبط لنمو الخلايا من النوع Vero، وأن الجرعة ٠,٠٣ ميكروجرام /سم٣ تسبب أيضا أعلى تأثير مثبط لنمو الخلايا من النوع BHK-21، ولم يظهر للسم الخام تأثير مثبط أو منشط لنمو الخلايا من النوع BGM عند أي جرعة من الجرعات المستخدمة. وقد تم اختبار كل مكون من مكونات السم الخام على حده على نوعي الخلايا Vero و BGM ووجد أن المكون الرابع له تأثير منشط لانقسام الخلايا ويسبب زيادة معنوية في عدد الخلايا مقارنة بالمجموعة الضابطة.