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異藻藍蛋白抑制腸病毒 71 型所引起的細胞凋亡

Inhibition of Enterovirus 71-induced Apoptosis by Allophycocyanin Isolated From a Blue-Green Alga Spirulina platensis.

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內容簡介:

腸病毒感染在孩童身上具有高發病率及致死率，但至今仍無有效的治療方法出現。在本研究中，首先發現一種純化自藍綠藻的蛋白鍵結色素－異藻藍蛋白（allophycocyanin）具有抑制腸病毒 71 型的能力。不論是在人類橫紋胚胎肌肉瘤細胞（human rhabdomyosarcoma cells）或是非洲綠猴腎上皮細胞（African green monkey kidney cells）中，異藻藍蛋白均可中和腸病毒 71 型造成的細胞病變（cytopathic effect）。在中和非洲綠猴腎上皮細胞病變的實驗中，異藻藍蛋白濃度 0.045+/-0.012 μM，即可達到 50% 中和效果（IC_{50}=0.045+/-0.012 μM）。而異藻藍蛋白對人類橫紋胚胎肌肉瘤細胞及非洲綠猴腎上皮細胞造成細胞毒性的濃度分別爲 1.653 +/- 0.003 μM及 1.521 +/- 0.012 μM。病毒斑抑制分析顯示：在病毒吸附細胞前加入異藻藍蛋白，濃度僅 0.056 +/- 0.007 μM，即可達到 50% 抑制病毒斑生成效果；在病毒吸附後再加入異藻藍蛋白，濃度需 0.101 +/- 0.032 μM，達到 50% 抑制效果。比較病毒感染細胞前後加入異藻藍蛋白造成的保護效果，顯示在感染前處理會較有效。異藻藍蛋白同時也能有效延遲病毒RNA在被感染細胞中生成；並且緩和被感染細胞的細胞凋亡（Apoptosis）過程。總結以上結果顯示：異藻藍蛋白具有抑制病毒的能力，並且有潛力被發展成一種抑制腸病毒 71 型的藥劑。

前言

腸病毒和流行性感冒病毒都是以核糖核酸（Ribonucleic acid，RNA）爲主要遺傳物質的病毒。腸病毒包含了70種不同的型態,包括脊髓灰質炎病毒,克沙奇病毒A,B,伊科拉病毒,以及其他編號之腸病毒。臨床證明，腸病毒的感染範圍從輕微的夏季感冒到嚴重的神經以及心血管疾病均有。在１９９８年，台灣爆發腸病毒７１型的大流行，許多小孩因此得了手足口症，腦膜炎，腦炎及小兒麻痺，其中又有８０個小孩死亡。在１９９８的腸病毒大流行之後，腸病毒便持續地在台灣流行且接二連三地造成許多死亡，

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所以非常急需發展腸病毒７１型的疫苗。

Pleconaril 已被證實體外實驗中藉著干擾病毒接受器的附著可抑制鼻病毒 (rhinovirus) 和一些腸病毒 (McKinlay,1993) 而且在臨床證明中也發現 Pleconaril 很有潛力對抗腸病毒７１型。但是 Pleconaril 卻無法中和在台灣爆發的腸病毒７１型所引起的細胞死亡，這個發現顯示了需要由本土資源中去發展特定對抗台灣腸病毒７１型的材料。

異藻藍蛋白是一種從藻類中萃取出的藍色螢光蛋白，這種螢光物質是從微細螺旋藻中萃取出的藻螢光蛋白的一員。從藍綠藻中抽出的ＣＰＣ曾被報導有抗氧化及抗炎的物質(Gonzalez,1999;Romay,1999)，另外也有報導從藻類中所萃取出來的物質具有抗病毒之效果(Carlucci,1997;Ayehunie,1998)，在此次研究中，我們將會探討APC在細胞實驗中對於抗腸病毒的機制，在對宿主細胞無毒害的濃度之下，發現APC即有抑制腸病毒７１型所導致的細胞死亡現象，病毒斑生成現象，以及病毒所引起的細胞凋亡。因此異藻藍蛋白很有潛力可被用來開發爲抗腸病毒７１型的藥劑。

實驗結果

(1)細胞的毒性

我們首先測試ＡＰＣ對細胞的毒性。將濃度 0.095－0.052 μM 的 APC 加入橫紋胚胎肌肉瘤細胞和非洲綠猴腎上皮細胞中 48 小時，在這 48 小時中沒有減少細胞活性。在濃度低於１ μM 時，細胞的形狀及密度也沒有明顯的改變。估計對於橫紋胚胎肌肉瘤細胞活性有影響的濃度是 1.653+/-0.003 μM，對非洲綠猴腎上皮細胞活性有影響的濃度是在 1.521+\-0.012 μM(圖 1)

(2)中和ＥＶ７１型所導致的細胞病變

在圖２可以看出ＡＰＣ對於腸病毒７１型的抑制效果。首先可看出被腸病毒７１型感染的非洲綠猴腎上皮細胞呈現類淚滴狀的細胞病變(2B)，當將濃度爲 0.238 μM 的ＡＰＣ加入時，發現這種細胞病變會被完全中和(2F)，且細胞型態在此濃度下並不會被改變(2E)。而在被腸病毒感染的橫紋胚胎肌肉瘤細胞也可以用同樣 APC 濃度中和。經過實驗，有效濃度約爲 0.045+\-0.012 μM，此外ＡＰＣ對克沙奇病毒 A16 亦有抑制效果。

(3)減少病毒斑的形成

爲了確定ＡＰＣ對於腸病毒７１型的抑制效果，我們繼續做病毒斑的分析實驗。在

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圖３Ａ中，ＡＰＣ抑制了被腸病毒７１型感染的非洲綠猴腎上皮細胞的病毒斑。經過一連串的濃度測試，在細胞被感染前的有效抑制濃度爲 0.056+\-0.007 μM，而在細胞被感染後的有效抑制濃度爲 0.101+\-0.032 μM。圖３Ｃ表現了ＡＰＣ在細胞被病毒感染前減少病毒斑形成的效果比細胞被病毒感染後來的有效。

(4)延緩病毒ＲＮＡ在感染細胞內的合成

在圖４中，用 VP1 RNA 可以很明顯的偵測出非洲綠猴腎上皮細胞在被感染病毒８小時後的病毒反應，然而在同樣的時間點，另一組有添加ＡＰＣ的感染細胞則很難偵測到病毒反應，這表現出ＡＰＣ可延緩病毒ＲＮＡ在感染細胞內的合成。

(5)抑制ＥＶ７１型所導致的細胞凋亡現象

爲了能更了解 APC 保護細胞不使其凋亡的機制，我們做了３個實驗。
1.在控制組中，橫紋胚胎肌肉瘤細胞在加入抗生素經過２４小時後，可明顯發現破碎的 DNA 片段，但是細胞中若加入 APC 則不會看見破碎的 DNA 片段。
2.Annexin-V-FLUO 檢驗腸病毒７１型所引起的細胞凋亡，由圖６的綠色螢光部分代表被腸病毒７１型破壞的橫紋胚胎肌肉瘤細胞膜，但加入 APC 之後，綠色螢光的密度降低，代表受到破壞的細胞膜減少
3.流式細胞儀檢驗腸病毒７１型所引起的細胞凋亡，但當橫紋胚胎肌肉瘤細胞加入 APC 之後，便回覆正常狀況(A,C,E 對照組;B 感染細胞;D,F 爲感染細胞,在 APC 不同濃度下恢復到未受感染的狀態)

討論

經過以上探討，我們證實一種純化自藍綠藻的蛋白鍵結色素－異藻藍蛋白（allophycocyanin）具有抑制腸病毒 71 型的能力。不論是在人類橫紋胚胎肌肉瘤細胞（human rhabdomyosarcoma cells）或是非洲綠猴腎上皮細胞（African green monkey kidney cells）中，異藻藍蛋白均可中和腸病毒 71 型造成的細胞病變（cytopathic effect）。

此外，從許多篇的學術文章中也出現許多有關藻類萃取物對於抑制及預防病毒的結果，例如愛滋病病毒，(Lynch et al.,1993,1994)，因此從藍綠藻中萃取出的其他類似蛋白質或是新的複合物，在抗病毒的功效上都是非常值得去開發的。總結以上結果顯示：異藻藍蛋白具有抑制病毒的能力，並且有潛力被發展成一種抑制腸病毒 71 型的藥劑。

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Abstract:
Enterovirus 71 infection causes significant morbidity and mortality in children, yet there is no effective treatment. In this study, a protein-bound pigment, allophycocyanin purified from blue-green algae is first reported to exhibit anti-enterovirus 71 activity. Allophycocyanin neutralized the enterovirus 71-induced cytopathic effect in both human rhabdomyosarcoma cells and African green monkey kidney cells. The 50% inhibitory concentration of allophycocyanin for neutralizing the enterovirus 71-induced cytopathic effect was approximately 0.045 +/- 0.012 μ M in green monkey kidney cells. The cytotoxic concentrations of allophycocyanin for rhabdomyosarcoma cells and African green monkey kidney cells were 1.653 +/- 0.003 μ M and 1.521 +/- 0.012 μ M, respectively. A plaque reduction assay showed that the concentrations of allophycocyanin for reducing plaque formation by 50% were approximately 0.056 +/- 0.007 μ M and 0.101 +/- 0.032 μ M, when allophycocyanin were added at the state of viral adsorption and post-adsorption, respectively. Antiviral activity was more efficient in cultures treated with allophycocyanin before viral infection compared with that in the cultures treated after infection. Allophycocyanin was also able to delay viral RNA synthesis in the infected cells and to abate the apoptotic process in enterovirus 71-infected rhabdomyosarcoma cells with evidence of characteristic DNA fragmentation, decreasing membrane damage and declining cell sub-G1 phase. It is concluded that allophycocyanin possesses antiviral activity and has a potential for development as an anti-enterovirus 71 agent. J. Med. Virol. 70:119-125, 2003. Copyright 2003 Wiley-Liss, Inc.

INTRODUCTION

Enterovirus 71 is a positive-stranded RNA virus of the genus Enterovirus within the family Picornaviridae. Enteroviruses comprise nearly 70 distinct serotypes, including the polioviruses, coxsackieviruses A and B, echoviruses, and the “numbered enteroviruses.” Clinical manifestations of enterovirus infetion range from a mild “summer cold” to neurologic and cardiovascular disorders. In 1998, there was a large enterovirus 71 outbreak in Taiwan. Many children became ill with hand, foot, and mouth disease, aseptic meningitis/encephalitis or acute flaccid paralysis, and there were nearly 80 fatalities. After the 1998 outbreak, enterovirus 71 was isolated continuously throughout the island, and many severe diseases as well as fatal cases caused by enterovirus 71 have been reported. Thus there is some urgency to develop an anti-enterovirus 71 agent.

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Pleconaril, an anti-picornavirus capsid-binding agent, has been shown to inhibit rhinovirus and some enteroviruses in vitro by interfering with capsid-receptor binding, and its potential against enterovirus 71 has been assessed by clinical trial. However, pleconaril was unable to neutralize the cytopathic effects in cultured cells induced by enterovirus 71 isolates from the 1998 outbreak in Taiwan. This finding suggests the need to develop specific anti-enterovirus 71 agents using material from local resources.

Allophycocyanin is a red fluorescent protein that can be isolated from the marine algae *Spirulina platensis*. It has been reported that C-phycocyanin from blue-green algae possesses anti-oxidant and anti-inflammatory properties. Extracts from some algae also have been demonstrated to have the anti-viral activity. In this study, we examined the in vitro anti-enteroviral mechanism of allophycocyanin. At concentrations nontoxic to the host cells, allophycocynin was found to inhibit enterovirus 71-induced cytopathic effects, viral plaque formation, and viral-induced apoptosis. This algal protein can be developed potentially as an anti-enterovirus 71 agent.

RESULTS

Cytotoxicity

We first evaluated the cytotoxicity of allophycocyanin. Confluent rhabdomyosarcoma and African green monkey kidney cells monolayers treated for 48 hours with allophycocyanin at concentrations of 0.095 to 0.952 μM did not show significant reduction of cell viability (fig. 1.) No visible changes in cell morphology or cell density were observed at concentrations below 1 μM. The estimated concentrations that reduced cell viability by 50%, that is, the CC_{50} were 1.653 ±0.003 μM and 1.521 ±0.012 μM for rhabdomyosarcoma and African green monkey kidney cells, respectively.

Neutralization of Enterovirus 71-Induced Cytopathic Effect

The inhibition of enterovirus 71-infected cytopathic effect is shown in Figure 2. Enterovirus 71-infected African green monkey kidney cells exhibited a typical tear-like cytopathic effect (Fig. 2B). This cytopathic effect was added (Fig. 2F). There was no obvious change in cell morphology when the cell culture medium contained the same concentration of allophycocyanin (Fig. 2E). Enterovirus 71-induced cytopathic effect in

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rhabdomyosarcoma cells was also neutralized by allophycocyanin. The antiviral activity of allophycocyanin for coxsackievirus A16 was also examined. The result indicated that allophycocyanin neutralized the coxsackievirus A16-induced cytopathic effect in rhabdomyosarcoma cells.

Reduction of Plaque Formation

To confirm the anti-enterovirus 71 activity of allophycocyanin, a plaque reduction assay was carried out. As shown in Figure 3A, allophycocyanin inhibited plaque formation in enterovirus 71-infected African green monkey kidney cells. Serial concentrations of allophycocyanin were tested to determine the amount needed to reduce plaque formation. Figure 3C shows that allophycocyanin inhibited plaque formation more efficiently when it was added before viral cell adsorption than when it was added after viral cell adsorption.

Delay of Viral RNA Synthesis

As shown in Figure 4, viral VP1 RNA can be clearly detected by RT-PCR in the infected African green monkey kidney cells at 8 hours post-adsorption. However, at the same time point (8 hours post-adsorption), viral RNA was barely detected when the cells was treated with allophycocyanin. It appears that allophycocyanin delays viral RNA synthesis in the infected cells.

Inhibition of EV71-Incuced Apoptosis

To understand further the mechanism by which allophycocyanin prevented cell death in enterovirus 71-infected cells, several experiments that involoved apoptosis were carried out.

1. As a positive control, DNA fragmentation can be observed when rhabdomyosarcoma cells are treated with actinomycin D for 24 hours (fig. 5, lane 3). When allophycocyanin was added, no DNA fragmentation was observed in enterovirus 71-infected cells (Fig. 5, lane7).

2. Enterovirus 71-induced apoptosis was also examined by the Annexin-V-FLUOS binding assay. When the cell was treated with allophycocyanin the fluorescent intensity was decreased indicating reduced cell membrane damage.

3. Enterovirus 71-induced apoptosis was also analyzed by flow cytometry (Fig. 7). When

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enterovirus 71 was added, the rhabdomyosarcoma cells showed a peak in the sub-G1 phase that is characteristic of cells undergoing apoptosis. When allophycocyanin alone was added to rhabdomyosarcoma cells, no such peak was observed (Fig. 7C,E).

In summary, allophycocyanin inhibited enterovirus 71-induced apoptosis in rhabdomyosarcoma cells, including characteristic nucleosomal fragmentation, decreasing membrane damage, and the appearance of sub-G1 phase.

Fig. 1.

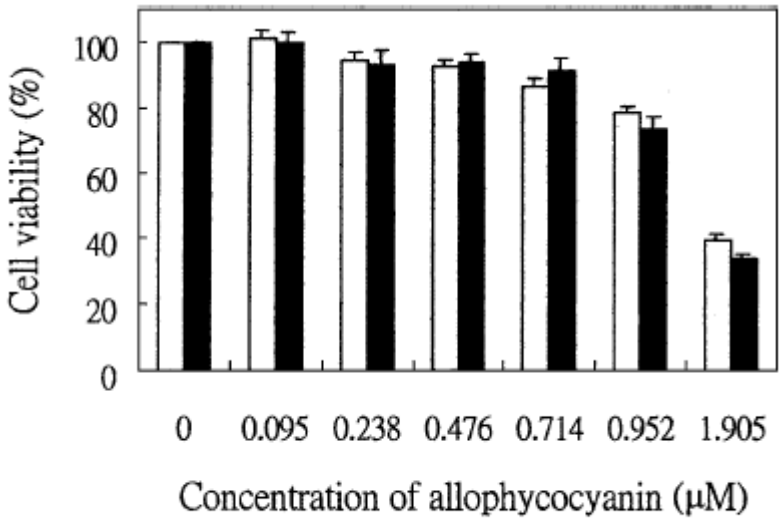

Fig. 1. The effects of allophycocyanin on cell viability. The open bars indicate cytotoxicity for rhabdomyosarcoma cells, and the filled bars indicate the cytotoxicity for African green monkey kidney cells. Each experiment involved triplicate wells per concentration.

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Fig. 2.

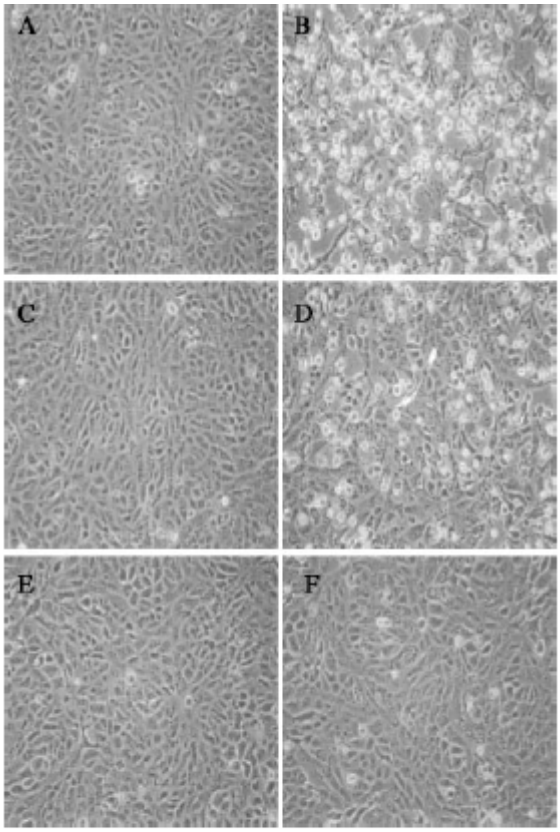

Fig. 2. Inhibition of enterovirus 71-induced cytopathic effect in culture cells. African green monkey kidney cells were cultured in 6-well plates to reach confluence. Cells were infected with virus (1 m.o.i.) for 36 hours. Allophycocyanin was added at the stage of viral adsorption. The cytopathic effect was observed under microscopy (×40). (**A**) Mock infection. (**B**) Infection without allophycocyanin. (**C**) Mock infection plus 0.119 μM of allophycocyanin. (**D**) Infection plus 0.119 μM of allophycocyanin. (**E**) Mock infection plus 0.238 μM of allophycocyanin. (**F**) Infection plus 0.238 μM of allophycocyanin.

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Fig. 3.

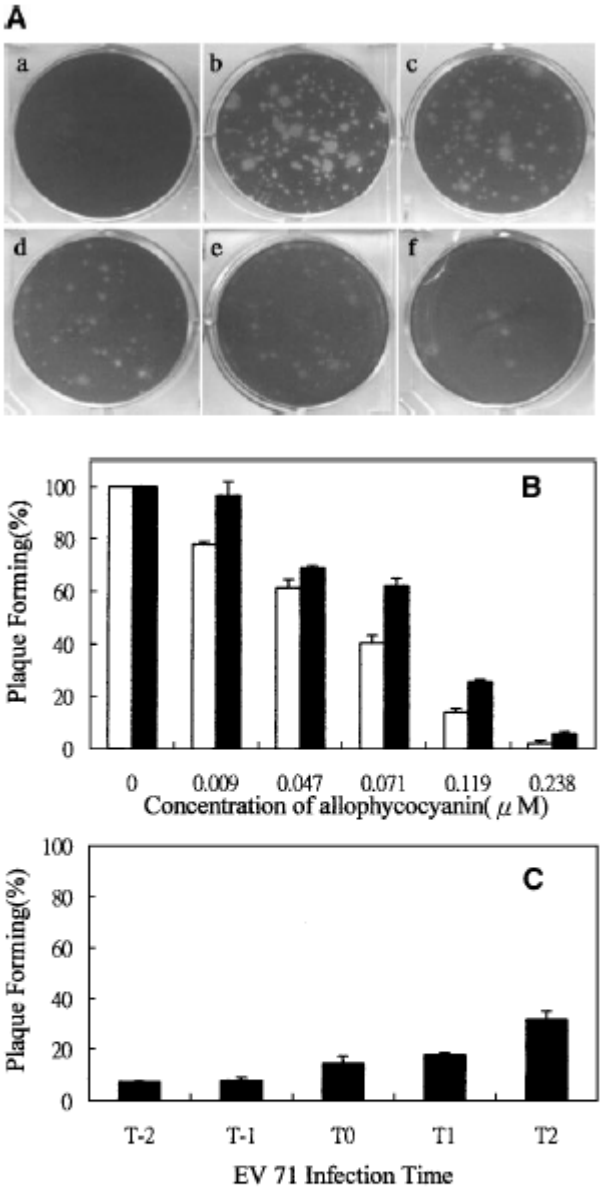

Fig. 3. Inhibition of plaque formation. (**A**) The effect of allophycocyanin on plaque formation. Allophycocyanin was added to African green monkey kidney cells at the stage of viral adsorption. (a) Mock infection. (b) Infection without allophycocyanin. (c–f) Infection and treatment of 0.047 μM, 0.071 μM, 0.119 μM, and 0.238 μM of allophycocyanin, respectively. (**B**) Quantification of viral plaques when treated with allophycocyanin at the adsorption stage (open bar) or post-adsorption (filled bar). (**C**) Plaque reduction at different time point of treatment. T-2: Allophycocyanin was added to African green monkey kidney cells before 2 hours of viral adsorption. T-1: Allophycocyanin was added to African green monkey kidney cells before 1 hour of viral adsorption. T0: Allophycocyanin was added to African green monkey kidney cells at the same time of viral adsorption. T1: Allophycocyanin was added to African green monkey kidney cells after 1 hour of viral adsorption. T2: Allophycocyanin was added to African green monkey kidney cells after 2 hours of viral adsorption. 0.119 μM of allophycocyanin was chosen to treat with cells. The percentage of plaque formation was relative to cell control (no allophycocyanin treatment).

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Fig. 4.

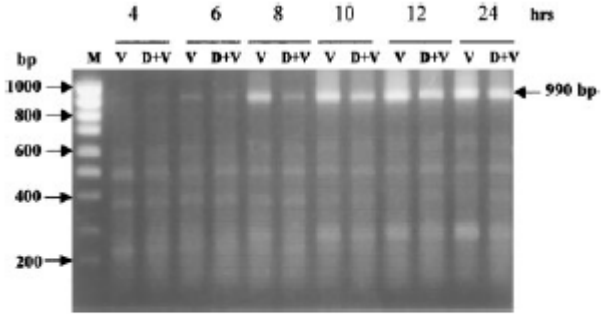

Fig. 4. Delay of Viral RNA synthesis by allophycocyanin. Total RNA was extracted at 4, 6, 8, 10, 12, and 24 hours post-viral adsorption. Allophycocyanin was added at the stage of viral adsorption. The same amount of total RNA (0.5 μg) extracted from infected-cells was used in each RT-PCR experiment, and a volume of 5 μl reaction solution was applied in each gel well. M: 100 bp molecular weight marker. V: virus infection without APC treatment. D+V: virus infection plus APC treatment. The arrow indicates the RT-PCR product from viral RNA. The size of the VP1 from RT-PCR is 990 bp.

Fig. 5.

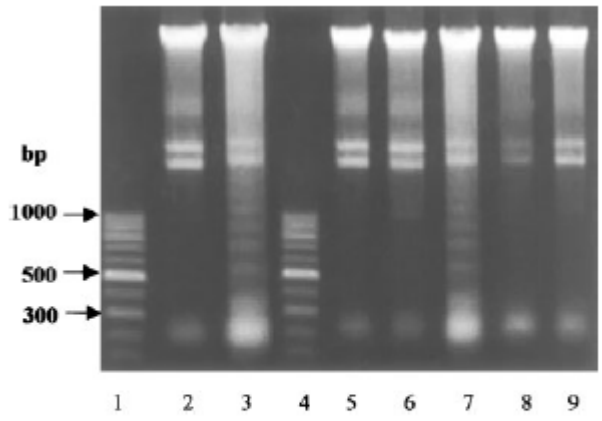

Fig. 5. Inhibition of enterovirus 71-induced internucleosomal DNA fragmentation. Rhabdomyosarcoma cells harvested at 48 hours post-infection were processed to assess DNA fragmentation. **Lanes 1** and **4**, 1 kb molecular weight marker. Mock-infected cells were used as a negative control (**lane 2**). Actinomycin D-treated cells were used a positive control for apoptosis (**lane 3**). **Lane 5** and **6**, cells treated with 0.071 μM and 0.119 μM of allophycocyanin, respectively. **Lane 7**, rhabdomyosarcoma cells infected with enterovirus 71 (m.o.i. = 1). **Lane 8** and **9**, infected cells plus 0.071 μM and 0.119 μM of allophycocyanin.

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Fig. 6.

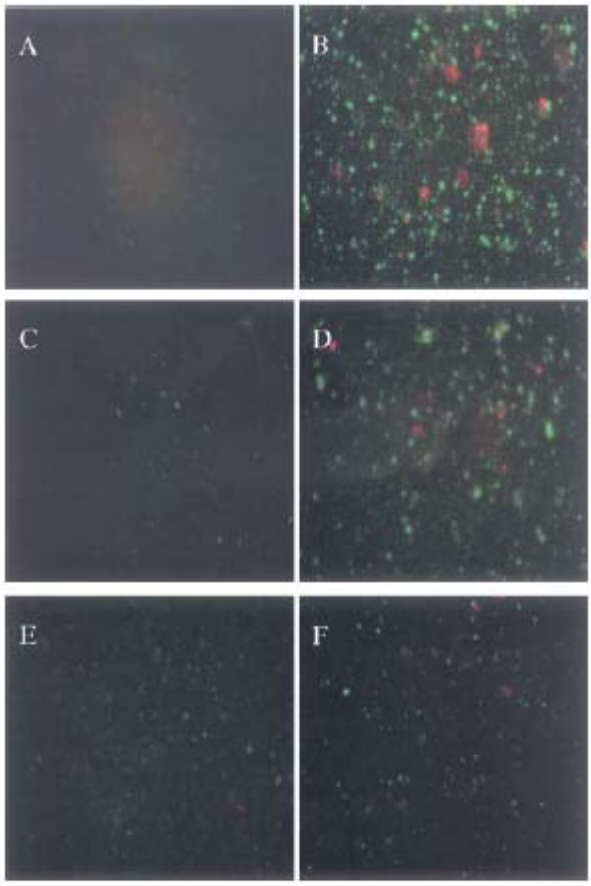

Fig. 6. Inhibition of enterovirus 71-induced apoptosis by Annexin-V-Fluos binding assay. (**A**) Mock-infected cells. (**B**) Cells infected with enterovirus 71 (1 m.o.i.). (**C**) Cells treated with 0.048 μM of allophycocyanin. (**D**) Infected cells treated with 0.048 μM of allophycocyanin. (**E**) Cells treated with 0.071 μM of allophycocyanin. (**F**) Infected cells treated with 0.071 μM of allophycocyanin.

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Fig. 7.

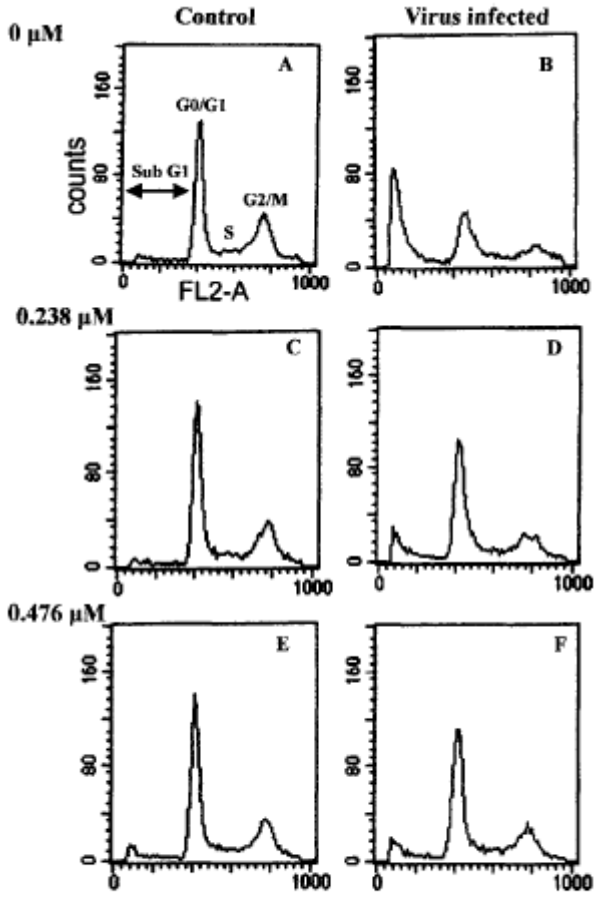

Fig. 7. Flow cytometric analysis of enterovirus 71-infected rhabdomyosarcoma cells. (**A**) Mock-infected cells. (**B**) Cells infected with enterovirus 71 (1 m.o.i.). (**C**) Cells treated with 0.238 μM of allophycocyanin. (**D**) Infected cells treated with 0.238 μM of allophycocyanin. (**E**) Cells treated with 0.476 μM of allophycocyanin. (**F**) Infected cells treated with 0.476 μM of allophycocyanin. FL2-A represents the intensity of propidium iodine.

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