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

Shin-Ru Shih,[1] Kun-Nan Tsai,[1] Yi-Shuane Li,[1] Chuang-Chun Chueh,[2] and Err-Cheng Chan[1]*

[1]*School of Medical Technology, Chang Gung University, Tao-Yuan, Taiwan*
[2]*Far-East Biotechnology Corp., Taipei, Taiwan*

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 μM in green monkey kidney cells. The cytotoxic concentrations of allophycocyanin for rhabdomyosarcoma cells and African green monkey kidney cells were 1.653 ± 0.003 μM and 1.521 ± 0.012 μM, respectively. A plaque reduction assay showed that the concentrations of allophycocyanin for reducing plaque formation by 50% were approximately 0.056 ± 0.007 μM and 0.101 ± 0.032 μM, 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.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: cytopathic effect; antiviral activity; phycobiliprotein; cell cycle

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 infection 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 [Chang et al., 1999; Ho et al., 1999; Shih et al., 2000]. 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 [Wang et al., 2002]. Thus there is some urgency to develop an anti-enterovirus 71 agent.

Pleconaril, an anti-picornavirus capsid-binding agent, has been shown to inhibit rhinovirus and some enteroviruses in vitro by interfering with capsid-receptor binding [McKinlay, 1993], and its potential against enterovirus 71 has been assessed by clinical trial [Romero, 2001; Turner, 2001; Rotbart, 2002]. However, pleconaril was unable to neutralize the cytopathic effects in

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*Correspondence to: Dr. Err-Cheng Chan, School of Medical Technology, Chang Gung University, 259, Wen-Hua 1st Road, Kwei-Shan, Tao-Yuan 333 Taiwan.
E-mail: chanec@mail.cgu.edu.tw

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cultured cells induced by enterovirus 71 isolates from the 1998 outbreak in Taiwan [Shia et al., 2002]. 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*. This fluorochrome is a member of the phycobiliprotein family that can be found in blue-green algae, red algae, and cryptomonads. Allophycocyanin has a molecular weight of 104,000 and consists of two distinguishable protein subunits designated α and β, which contain at least three covalently attached bilin chromophores, open chain tetrapyrroles with no metal complexes [Duerring et al., 1991]. It has been reported that C-phycocyanin from blue-green algae possesses antioxidant and anti-inflammatory properties [Gonzalez et al., 1999; Romay et al., 1999]. Extracts from some algae also have been demonstrated to have the antiviral activity [Carlucci et al., 1997; Ayehunie et al., 1998; Hoshino et al., 1998; Fabregas et al., 1999; Serkedjieva et al., 2000]. 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.

MATERIALS AND METHODS

Preparation of Allophycocyanin

Allophycocyanin was kindly given by Far-East Biotechnology, Inc. (Taiwan, Taipei) and was purified for further use. In brief, approximately 10 g of *Spirulina* powder were suspended in 500 mL of 0.1 M potassium phosphate buffer (pH 7.0), containing 100 μg/mL lysozyme, 10 mM EDTA, and 2 mM PMSF. The cellular lysis was put in a shaker overnight at 37°C. The suspension was first centrifuged for 1 hour at 48,400g to remove cell debris and was then centrifuged at 110,000g for 90 minutes in order to remove any remaining precipitate. The crude protein extract was precipitated with 50% (w/v) saturated ammonium sulphate and then centrifuged for 30 minutes at 48,400g to obtain a pellet. The pellet was subsequently dissolved in a 5 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA, and dialyzed against the same buffer. The phycobiliproteins were separated by DEAE-cellulose chromatography (DE-52, Whatman; dimensions 30 cm × 4 cm). The column was developed with a potassium phosphate gradient of 5 mM to 300 mM at flow rate of 55 mL/h. The pooled fractions, containing a mixture of allophycocyanin and C-phycocyanin, were concentrated by ultrafiltration using a Diaflo PM30 membrane (Amicon, Danvers, MA) and dialyzed against a 5 mM potassium phosphate buffer (pH 7.5). The hydroxyapatite column-step was then carried out twice. All steps were done at 4°C in the dark. All chemical were analytical grade, and all buffer solutions contained 0.02% (w/v) sodium azide. At the end of the second run the purity of the pooled fractions was checked by SDS/PAGE, fluorescence and absorption spectra. For sequencing, the subunits were separated by reversed phase HPLC. N-terminal sequencing was done by automated Edman degradation in a Porton LF 3600 gas phase sequencer from Beckman, equipped with a microbore System Gold for the identification of phyenylthiohy dantoin-amino acids (Pth-Xaa).

Cells and Viruses

Human rhabdomyosarcoma cells and African green monkey kidney cells were purchased from the American Type Culture Collection (ATCC) and their 20th culture passages were used to propagate viruses. Virus titer was measured by a plaque assay using African green monkey kidney cells. Enterovirus 71-2231-TW was isolated in the 1998 outbreak and supplied by the Clinical Virology Laboratory of Chang Gung Memorial Hospital, Taiwan.

Neutralization Test

This assay was used to test the antiviral efficacy of allophycocyanin by measuring the inhibition of cytopathic effect induced by enterovirus 71 on rhabdomyosarcoma cells. The 96-well tissue culture plates were seeded with 200 μL of rhabdomyosarcoma cells at a concentration of 3×10^5 cells/mL in DMEM with 10% FBS. The plates were incubated for 24 to 30 hours at 37°C and were used at about 90% confluence. Virus (100 $TCID_{50}$) mixed with different concentrations of allophycocyanin (0.022–0.238 μM) was added to the infected plates and incubated at 37°C for 1 hour. After adsorption, the infected cell plates were overlayed with 50 μl of DMEM plus 5% FBS and 2% DMSO. The plates was wrapped in parafilm and incubated at 37°C for 64 hours. At the end of incubation, the plates were fixed by the addition of 100 μL of 0.5% glutataldehyde, and stained with 0.1% crystal violet for 15 minutes at room temperature. The plates were washed and dried, and the density of the well was measured at 570 nm. The concentration required for allophycocyanin to reduce the virus-induced cytopathic effect by 50% relative to the virus control was expressed as IC_{50}.

Plaque Reduction Assay

The antiviral activity of test compounds was determined using a standard plaque reduction assay. In brief, African green monkey kidney cells in monolayers were infected at a virus concentration to give approximately 50 to 100 plaques per monolayer and compared with the virus control, which contained no test compound. Test compounds were diluted and included in the agar-medium overlay. Plates were incubated at 37°C for 48 hours and stained with crystal violet, and the plaques were counted. All experiments were carried out in triplicate and at least twice. The concentration required for a test compound to reduce the number of plaques by 50%, that is, IC_{50}, was then determined.

Cytotoxicity Assay

Test compounds at various concentrations were added to rhabdomyosarcoma and African green monkey kidney cells. The cells were then incubated at 37°C for 48 hours. After incubation, the cells were harvested and viable cells were counted by trypan blue staining. All experiments were undertaken in triplicate and at least twice. The concentration of a test compound required to reduce cell viability to 50% of the tested control culture was expressed as CC_{50}.

Detection of Viral RNA in EV71-Infected Cells

A total of 5×10^5 African green monkey kidney cells were seeded into 6-well plates to reach confluence and then challenged with virus (m.o.i. = 1). Total RNA was extracted from cells using the TRIzol reagent (Life Technologies, Bethesda, MD). Following phenol-chloroform extraction and isopropanol precipitation, the RNA pellet was washed and dried and dissolved in 10 μL of RNase-free water. The purity and concentration of RNA was determined both by measuring OD at A260/280 and by quantitating the ethidium-stained agarose gel bands. Reverse transcription and polymerase chain reaciton amplification were carried out by RT-PCR beads (Pharmacia Biotech, Sweden). The beads contained recombinant Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (FPLCpureTM) for cDNA synthesis, Taq DNA polymerase (2.0 units) for amplification, RNase inhibitor (RNA guard^{TM}, porcine), buffer (10 mM Tris-HCl, pH 9.0, 60 mM KCl, 1.5 mM $MgCl_2$), and 200 μM of each dNTPs. For RT-PCR, 0.5 μg of RNA and 50 pmoL of each primer were used. Each reaction was incubated in a Perkin Elmer Cetus DNA 480 thermal cycler by the following protocol: 30 minutes at 42°C, 5 minutes at 94°C, then 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, and an additional 15 minutes for elongation in the last cycle. RT-PCR products were examined by electrophoresis through 1% agarose gels with loading 5 μl in each gel well. The sequence of primers used for RT-PCR was as follow. 5′-GGAGATAGGGTGGCAGATGTGATTG-3′ (genomic positions: 2339–2463 of TW/2272/98) and 5′-GAGAGTGGTAATTGCTGTGCG-3′ (genomic positions: 3309–3329).

Cellular Death Assays

For analysis of DNA fragmentation, DNA was isolated using the Blood and Cell Culture DNA mini kit (Qiagen, Hiden, Germany) and analyzed by gel electrophoresis. Annexin-V-FLUOS binding assay (Roche, Mannheim, Germany) was also used for measuring the apoptotic cells. Cells stained with Annex-V-FLUOS were examined by fluorescent microscopy. For cell cycle analysis, control and virus-infected cells were stained with propidium iodide (Sigma Chemical Co., St. Louis, MO) and measured by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). CellQuest and Modfit LT 2.0 were used for data analysis.

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

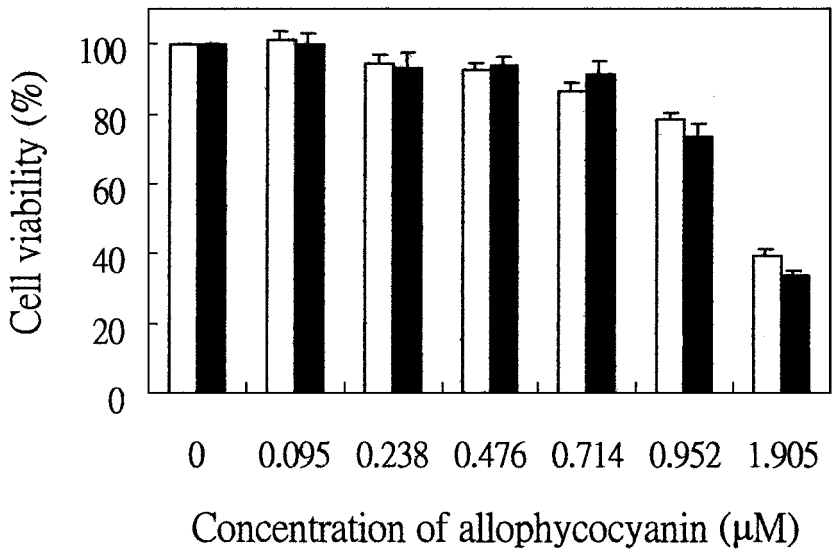

Fig. 1. The effects of allophycocyanin on cell viability. The open bars indicate cytotoxicity for rhabdomyosarcoma cells, and the filled bars indicate the cytotoxicity for African green monkey kidney cells. Each experiment involved triplicate wells per concentration.

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 completely neutralized when 0.238 μM of allophycocyanin 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 rhabdomyosarcoma cells was also neutralized by allophycocyanin (data not shown). Serial concentrations of allophycocyanin were tested for their ability to neutralize the enterovirus 71-induced cytopathic effect, and the IC_{50} was 0.045 ± 0.012 μM. 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 with IC_{50} of 0.51 ± 0.12 μM.

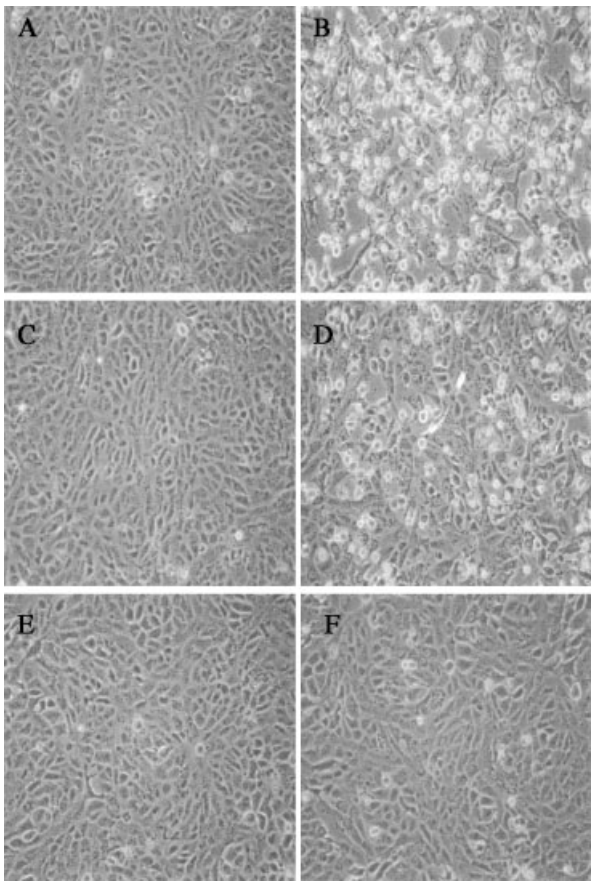

Fig. 2. Inhibition of enterovirus 71-induced cytopathic effect in culture cells. African green monkey kidney cells were cultured in 6-well plates to reach confluence. Cells were infected with virus (1 m.o.i.) for 36 hours. Allophycocyanin was added at the stage of viral adsorption. The cytopathic effect 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.

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. Approximately IC_{50} was 0.056 ± 0.007 μM and $0.101 + 0.032$ μM, respectively, when allophycocyanin was added at the stage of viral adsorption or post-adsorption (Fig. 3B). To understand further the timing of this effect, allophycocyanin was added to cells at different time points. 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-Induced Apoptosis

To understand further the mechanism by which allophycocyanin prevented cell death in enterovirus 71-infected cells, several experiments that involved apoptosis were carried out. As a positive control, DNA fragmentation can be observed when rhabdomyosarcoma cells are treated with actinomycin D for 24 hours (Fig. 5, lane 3). DNA extracted from mock-infected rhabdomyosarcoma cells was intact (Fig. 5, lane 2), whereas in enterovirus 71-infected cells the characteristic nucleosomal ladder appeared (Fig. 5, lane 7). When allophycocyanin was added, no DNA fragmentation was observed in enterovirus 71-infected cells (Fig. 5, lanes 8, 9). Allophycocyanin had no effect on DNA fragmentation of rhabdomyosarcoma cells without virus challenge (Fig. 5, lanes 5, 6). Enterovirus 71-induced apoptosis was also examined by the Annexin-V-FLUOS binding assay. Annexin-V-FLUOS binding assay is used to study the changes in plasma membrane, which are the characteristics involved at the middle stage of apoptosis. As shown in Figure 6, green fluorescence, which highlights the damaged cell membrane, can be clearly observed in enterovirus 71-infected rhabdomyosarcoma cells (Fig. 6B), whereas the fluorescent intensity was decreased when the cell was treated with allophycocyanin (Fig. 6D,F). Enterovirus 71-induced apoptosis was also analyzed by flow cytometry (Fig. 7). When enterovirus 71 was added, the rhabdomyosarcoma cells showed a

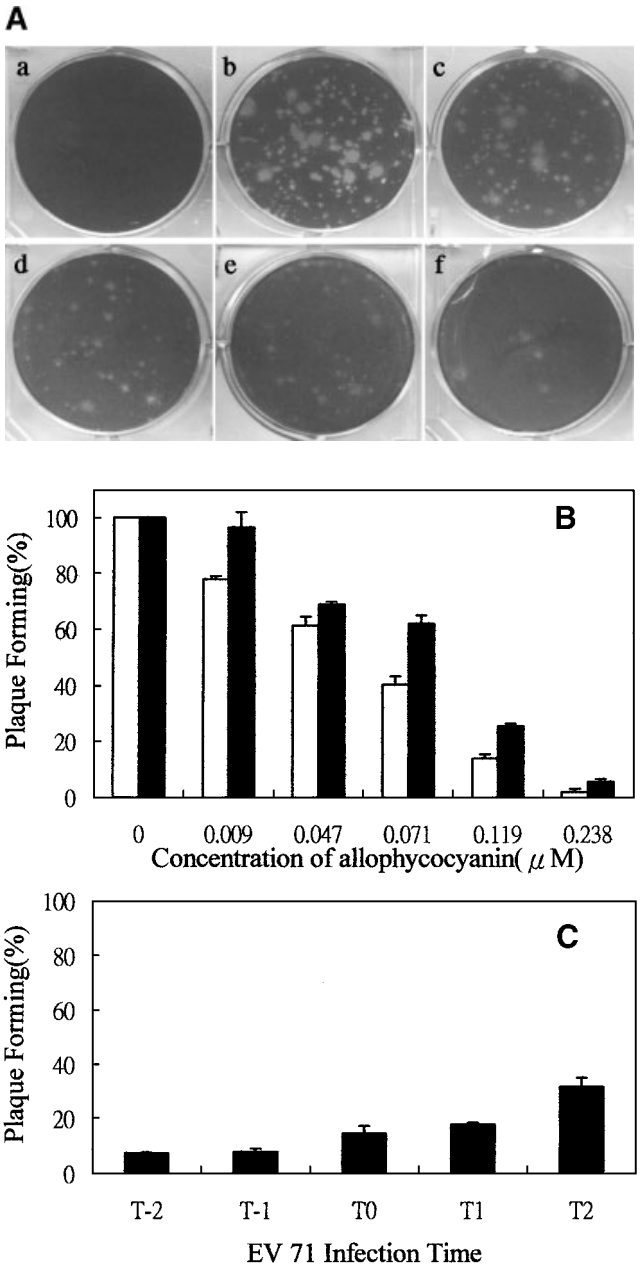

Fig. 3. Inhibition of plaque formation. (**A**) The effect of allophycocyanin on plaque formation. Allophycocyanin was added to African green monkey kidney cells at the stage of viral adsorption. (a) Mock infection. (b) Infection without allophycocyanin. (c–f) Infection and treatment of 0.047 μM, 0.071 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).

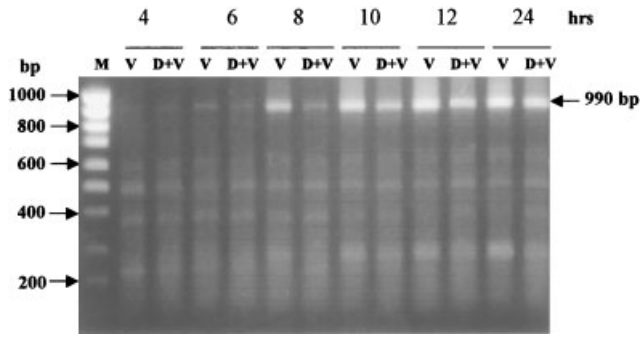

Fig. 4. Delay of Viral RNA synthesis by allophycocyanin. Total RNA was extracted at 4, 6, 8, 10, 12, and 24 hours post-viral adsorption. Allophycocyanin was added at the stage of viral adsorption. The same amount of total RNA (0.5 μg) extracted from infected-cells was used in each RT-PCR experiment, 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.

prominent new peak with a 100-fold increase in fluorescence (Fig. 7B). This peak represented the sub-G1 phase is characteristic of cells undergoing apoptosis. When allophycocyanin alone was added to rhabdomyosarcoma cells, no such peak was observed (Fig. 7C,E). The enterovirus 71-induced sub-G1 phase decreased when allophycocyanin was added to the cells (Fig. 7D,F). Taken together, allophycocyanin inhibited enterovirus 71-induced apoptosis in rhabdomyosarcoma cells, including characteristic nucleosomal fragmentation, decreasing membrane damage, and the appearance of sub-G1 phase.

DISCUSSION

We demonstrated that allophycocyanin inhibited enterovirus 71 replication and cell death using two cells

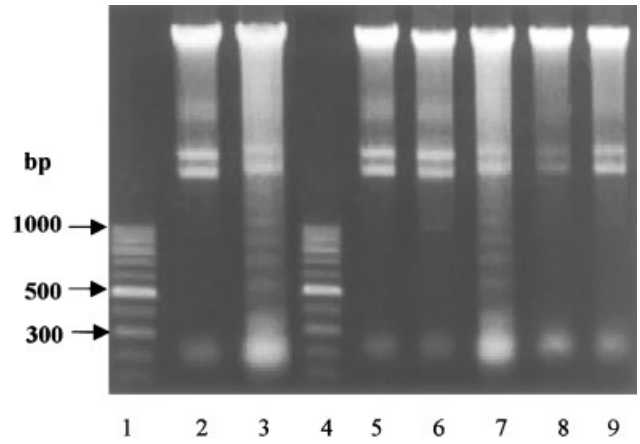

Fig. 5. Inhibition of enterovirus 71-induced internucleosomal DNA fragmentation. Rhabdomyosarcoma cells harvested at 48 hours post-infection were processed to assess DNA fragmentation. **Lanes 1** and **4**, 1 kb molecular weight marker. Mock-infected cells were used as a negative control (**lane 2**). 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.

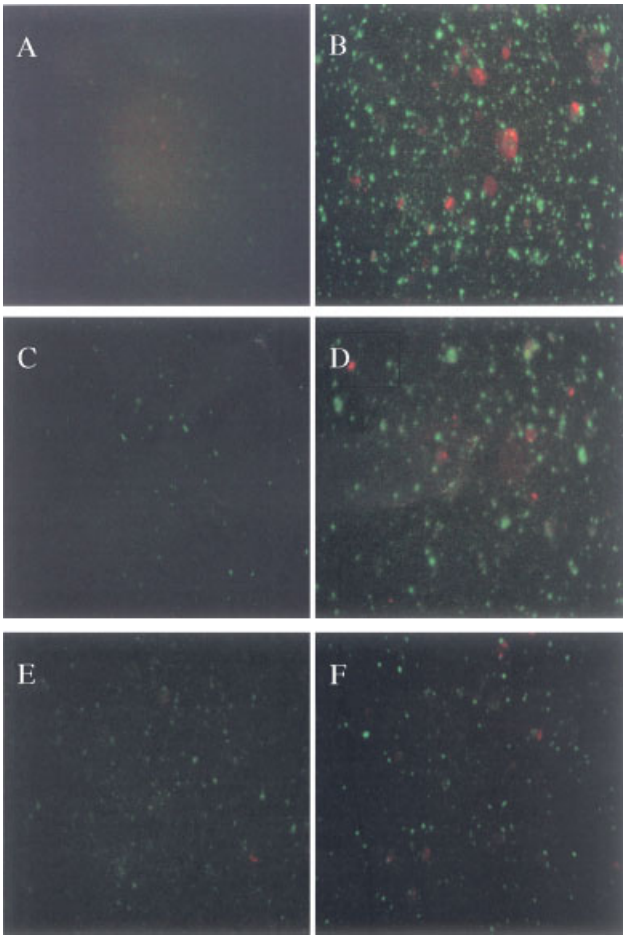

Fig. 6. Inhibition of enterovirus 71-induced apoptosis by Annexin-V-Fluos binding assay. (**A**) Mock-infected cells. (**B**) Cells infected with enterovirus 71 (1 m.o.i.). (**C**) Cells treated with 0.048 μM of allophycocyanin. (D) Infected cells treated with 0.048 μM of allophycocyanin. (**E**) Cells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

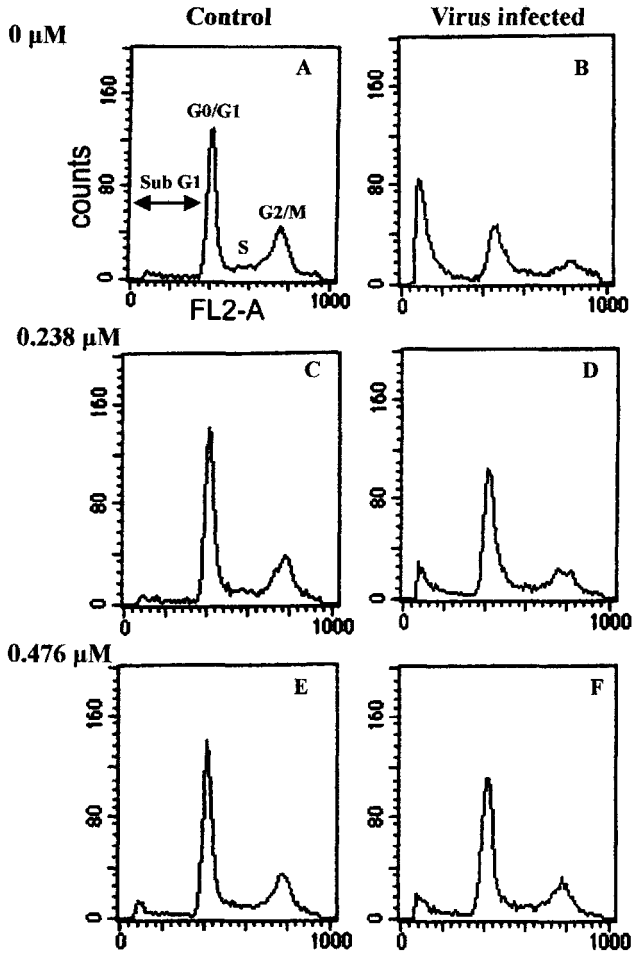

Fig. 7. Flow cytometric analysis of enterovirus 71-infected rhabdomyosarcoma cells. (**A**) Mock-infected cells. (**B**) Cells infected with enterovirus 71 (1 m.o.i.). (**C**) Cells treated with 0.238 μM of allophycocyanin. (**D**) Infected cells treated with 0.238 μM of allophycocyanin. (**E**) Cells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

line, rhabdomyosarcoma and African green monkey kidney cells. In these two cell lines, allophycocyanin inhibited enterovirus 71-induced cytopathic effect. Plaque formation in enterovirus 71-infected African green monkey kidney cells was inhibited when allophycocyanin was added. As shown in Figure 3C, a time course study revealed that the antiviral efficacy of allophycocyanin was higher when it was added before the virus entered the host cells than when it was added after the virus entered the host cells. These results suggest the allophycocyanin may interfere with a very early stage of viral replication such as virus adsorption and penetration.

Polysaccharide-containing extract of cultured blue-green algae have been shown to inhibit HIV viral replication through binding of the polysaccharides to the CD4 receptor, which results in the disruption of CD4-gp120 interaction [Lynch et al., 1994]. Direct effects of algal extracts on the reverse transcriptases of avian myeloblastoma virus and HIV have also been reported [Lau et al., 1993]. Recently, Boyd et al. [1996] isolated a novel antiviral protein from cultures of blue-green algae, *Nostoc ellipsosporum*. This 11 kDa antiviral protein inhibited in vitro replication and cytopathic effects of primate lentiviruses, including simian immunodeficiency virus and diverse laboratory strains of HIV-1 and HIV-2. Boyd suggested that the antiviral effect of 11 kDa protein results from the interaction of the antiviral protein with the gp120 component of the virus envelope glycoproteins [Boyd et al., 1996]. Whether a similar protein or other novel compounds are involved in the antiviral effect of the blue-green algae component is worthy of further investigation. In this study, a 104 kDa pigment protein derived from blue-green algae, allophycocyanin, was found to possess a better antiviral activity when it was added to the host cells before the virus entered the host cells. This finding may imply that the mode of action of this algal protein occurs in the stage of viral adsorption or penetration. Direct interaction or indirect pathways created by changing the property of the host cell membrane need to be further characterized. Although, the antiviral

mechanism of this algae protein remains to be elucidated, to our knowledge this is the first time that antivirus property has been described for allophycocyanin.

Enterovirus 71 induced apoptosis has been considered an important mechanism in disease pathogenesis [Lai et al., 2002]. In rhabdomyosarcoma cells, apoptosis was clearly observed upon infection with enterovirus 71 (Fig. 5). Apoptosis is a common cellular response to viral infection. It is a defense reaction to prevent generation and spread of the viral progeny. Although some viruses have evolved one or more ways to suppress this defense mechanism, other viruses trigger apoptosis in host cells at the late stages of infection to spread viral progeny [Teodoro and Branton, 1997; Roulston et al., 1999; Everett and Mcfadden, 2001]. Virus-induced apoptosis in nonrenewable cells, such as those in the central nervous system, where most neurons are postmitotic and therefore cannot be replaced, may result in an irreversible pathology. In 1998, there was a large outbreak of hand, foot, and mouth disease caused by enterovirus 71 in Taiwan. It infected more than 120,000 children and left nearly 80 dead. Post-mortem studies clearly showed that enterovirus 71 infected the central nervous system [Hsueh et al., 2000]. Furthermore, as mentioned above, apoptosis leads to the spread of viral progeny, which may cause viremia and severe central nervous system complications. In this study, an algal protein, allophycocyanin, was found to inhibit enterovirus 71-induced apoptosis, which may have a significant impact on protecting hosts from severe consequences associated with infection with enterovirus 71.

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