

Outbreak of fatal childhood viral infection in Sarawak, Malaysia in 1997: Inocula of patients' clinical specimens induce apoptosis *in vitro*

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Abstract

Identification of the aetiological **agent(s)** associated with an outbreak of fatal childhood viral infection in Sarawak, Malaysia, in mid 1997 remains elusive. It is reported here that African green monkey **kidney** (Vero) and human **monocytic (U937)** cells treated with inocula derived from clinical specimens of some of these fatal cases showed the presence of cellular genomic DNA degradation when the extracted DNA was separated by pulsed field gel electrophoresis (PFGE), oligonucleosomal, DNA ladders characteristic of apoptotic cells when the infected cells' DNA was separated by **agarose gel electrophoresis**, and apoptotic cellular DNA fragmentation when cells were stained using terminal deoxynucleotidyl transferase (**TdT**)-mediated **dUTP** nick-end labeling (TUNEL). These results suggest that inocula derived from the patients' clinical specimens contain factors which stimulate apoptotic cellular responses *in vitro*.

Key words: Apoptosis, Enterovirus, Malaysia, **Outbreak**, Sarawak

INTRODUCTION

In mid 1997, an outbreak of acute viral infection in various localities in Sarawak, Malaysia claimed 31 deaths among young children between the ages of 5 months to 6 years old. The sudden emergence and disappearance of the infection is puzzling and the origin of the infection has as yet not been ascertained. In most cases, it has been reported that the children died due to acute congestive heart failure and cardiovascular collapse consistent with a terminal event in acute viral myocarditis. Nonetheless, congestive heart failure as a terminal event in acute viral encephalomyelitis is also possible. **Occurrence** of the fatal infection in the midst of simultaneous outbreak of an enterovirus-associated hand, foot and mouth disease (HFMD), raised the possibility that an enterovirus is associated with the fatal outbreak. Enterovirus 71 (**EV71**) infection in particular was reported to cause fatal brainstem encephalomyelitis in at least 4 young children presenting with high fever and rapid deterioration of vital functions in Peninsula Malaysia in the following months subsequent to the outbreak in Sarawak.¹ In these later cases, EV71 was isolated, identified, and partially sequenced along with other EV71 isolates obtained from non-fatal HFMD patients

in Peninsula Malaysia and **Sarawak**.² **Outbreaks** of EV71 infection resulting in high mortality in children³ and severe neurological manifestations have been reported **worldwide**.^{4,5,6,7} However, a number of other enteroviruses including **Coxsackieviruses**⁸ and **Echoviruses**⁹ have also been associated with fatal infections but none of these viruses are known to cause outbreaks with high fatality rate comparable to that of the Bulgarian EV71 **outbreak**.³ While the enteroviruses especially EV71 is a possible etiologic agent of the fatal outbreak in Sarawak, efforts to isolate and identify viruses from the patients' specimens have been hampered by the inability to continuously propagate the virus in tissue culture cells. This is in contrast to the relative ease of isolating and propagating EV71 from patients who succumbed to the fatal EV71 brainstem encephalomyelitis seen in Peninsula **Malaysia**.^{1,2} Since EV71 was isolated and identified only from two of the Sarawak patients, this has led some investigators to suggest that perhaps other viruses were present in the patients **samples**.¹⁰ In the present report, we described detection of cytopathic effects and apoptotic cells in cell cultures treated with inocula derived from specimens of patients from the fatal outbreak in Sarawak.

MATERIALS AND METHODS***Cells, virus preparation, and infection***

Cells used in this investigation were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured and maintained as previously described." Patients' samples made available by Prof. Lam Sai Kit (Virology Diagnostic Unit, University Malaya Medical Center, Kuala Lumpur, Malaysia) were received as infected Vero cell culture supernatants on day 8 post-inoculation (PI) with the patients' specimens (Table 1). All samples were reported to have demonstrated cytopathic effects on day 8 PI. The supernatants were clarified to remove cell debris by sequential centrifugation at 10,000 x g and 40,000 x g. The supernatant was then filtered through 0.22 µm syringe filters and used as inoculum to infect freshly prepared Vero cells cultured in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark). In a typical experiment, approximately 100 µl of the virus inoculum or mock infecting fluid (similarly prepared as the virus inoculum except uninfected Vero cells were used), was added to 1 ml growth

medium. After 1-2 h incubation at 37°C the inoculum was removed and fresh growth medium was added. Cytopathic effects were monitored by observing the infected cell cultures under an inverted microscope (Zeiss, Germany).

Detection of apoptotic cells

Infected cells were scraped off the tissue culture flasks using sterile plastic cell scrapers on day 6 PI. Cells were sedimented by centrifugation at 800 x g and the pellet was washed with phosphate-buffered saline (PBS; 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 100 mM NaCl, 2.7 mM KCl) twice. Cells were placed on glass slides, air-dried, fixed in 4% paraformaldehyde for 25 min, washed with PBS and permeabilized using 0.5% Triton-X 100. Staining for apoptotic cells was performed using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) as previously described." Apoptotic cells' DNA labelled with fluorescein-12-dUTP appeared green when observed under a Zeiss Axiolab fluorescence microscope (Zeiss, Germany) using standard fluorescein excitation and emission filters.

TABLE 1: List of patients and their respective specimens collected for laboratory investigation. Samples received in viral transport medium were processed and used to inoculate Vero cells. Supernatants of these initial cultures after eight days post-inoculation were used as inocula in subsequent investigations.

Patient	Sample*	Diagnosis
SKV 1 (MY3/3)	CSF	Fatal
SKV 9 (MY10/1)	CSF	Fatal
SKV10 (MY4/4)	CSF	NA
SKV6b (MY1514)	CSF	Fatal
SKV2b (MY6/3)	Serum	Fatal
SKV 5 (MY 1/1)	Serum	Fatal
SKV 6 (MY15/3)	Serum	Fatal
SKV 8 (MY2/1)	Serum	Fatal
SKV11(MY 511)	Serum	Fatal
SKV4b(MY 1712)	Throat Swab	HFMD
SKV3b (MY 16/1)	Vesicle Swab	HFMD

*Initial sample of patients
NA - information not available

DNA analysis

Cells were sedimented by centrifugation at 800 x g and pellets were washed three times with homogenization buffer (0.01 M Tris-HCl pH 7.5, 0.15 M KCl, 0.002 M MgCl₂, 0.002 M CaCl₂). After the final washing, lysis solution consisting of 1% N-lauroylsarcosine, 0.2% sodium deoxycholate, and proteinase K (1 mg/ml) in L-buffer (10 mM Tris-HCl pH 7.6, 20 mM NaCl, 100 mM EDTA) was added. Samples were incubated at 50°C overnight and following the incubation and inactivation of proteinase K, RNase A (1 mg/ml) was added. Samples were incubated for additional 4 h at 50°C following which the samples were kept at 4°C until needed for electrophoresis. Agarose plugs for pulsed field gel electrophoresis (PFGE) were prepared by resuspending cells in pre-warmed (42°C) L-buffer and equal volume of molten (42°C) 1.5% (w/v) low melting point (LMP) agarose (InCert agarose, FMC Bioproducts, Rockland, ME, USA). The cell suspension was immediately transferred into a pre-chilled agarose plug mold (Bio-Rad Laboratories, Hercules, CA, USA) and the samples were refrigerated at 4°C for about 10 min. The agarose plugs were removed into universal bottles and treated with lysis solution as above. After washing three times with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) the agarose plugs were stored in 450 mM EDTA until needed for PFGE.

Electrophoresis of DNA

The presence of oligonucleosomal DNA ladder was detected by agarose gel electrophoresis. Equal amount of DNA was loaded into a 1.2% agarose gel and samples were electrophoresed at 80V for 1 h at room temperature in 0.5X TAE buffer (20 mM Tris-base, 10 mM sodium acetate, 5 mM EDTA). PFGE for separation of large DNA fragments was performed using the CHEF DR II (Bio-Rad Laboratories, Hercules, CA, USA). Samples were electrophoresed in 1.5% pulsed field certified agarose (w/v) gel using 0.5X TBE buffer (100 mM Tris-base, 90 mM boric acid, 25 mM EDTA) at 14°C. DNA was electrophoresed in two stages; 0.1 to 2.5 sec ramped pulse times, 200 V for 7 h followed by 0.2 to 13 sec switch time, 200 V for 16 h. Following electrophoresis, gels were stained with ethidium bromide (EtBr) and photographed under ultraviolet light illumination at 302 nm wavelength.

Detection of virus antigens

Staining for the presence of specific virus antigens except flaviviruses was performed using reagents purchased from Chemicon International Inc. (USA) following the manufacturer's protocols. The presence of flaviviruses antigens was examined using the 4G2 monoclonal antibodies (ATCC, USA).

Detection of viral genomes by the polymerase chain reaction

The polymerase chain reaction for detection of viruses was performed using oligonucleotide primers synthesized in accordance to the published literature.^{12,13,14} Total DNA and RNA of the treated cells were harvested using TRIzol™ Reagent (GIBCO BRL, Life Technologies Inc., USA). RT-PCR was performed using the Access RT-PCR System (Promega, USA). The PCR parameters were similar to those described in the literature from which the primer sequences were derived. DNA sequencing was done by ACGT Inc. (USA).

RESULTS

The cytopathic effects of the patients' inocula in Vero cells were observed in samples SKV2b, SKV5, SKV6, SKV8, and SKV9 beginning on day 3 post-infection (PI). Cells were noted to contract, become rounded, and floated off from the tissue culture flask surfaces. By day 4 PI, viral plaques began to be noted in samples of all patients except SKV3b and SKV4b (Figure 1). The plaques were distinct in comparison to that of the herpes simplex virus (Figure 1i). In most instances, each plaque appeared as though it originated as two foci adjacent to each other. By day 6 PI more than 70% of the Vero cells treated with inocula of patients SKV2b, SKV5, SKV6, and SKV9 had detached from the tissue culture flasks, whereas, samples from patients SKV3b, SKV4b, SKV8 and SKV11 had only <5%, <5%, -20% and -10% cells detached from the flasks, respectively (Figure 2).

In an effort to rapidly identify the possible virus causing the observed cytopathic effects, the infected cells were stained for the presence of respiratory viruses (adenoviruses, influenza virus, parainfluenza virus, respiratory syncytial virus), enteroviruses (Coxsackievirus B1, B2, B3, B4, B5, B6, A24; echovirus 4, 6, 9, 11, 30, 34; enterovirus 70 and 71; poliovirus 1, 2, 3) and flaviviruses (dengue 1, 2, 3, 4 and Japanese

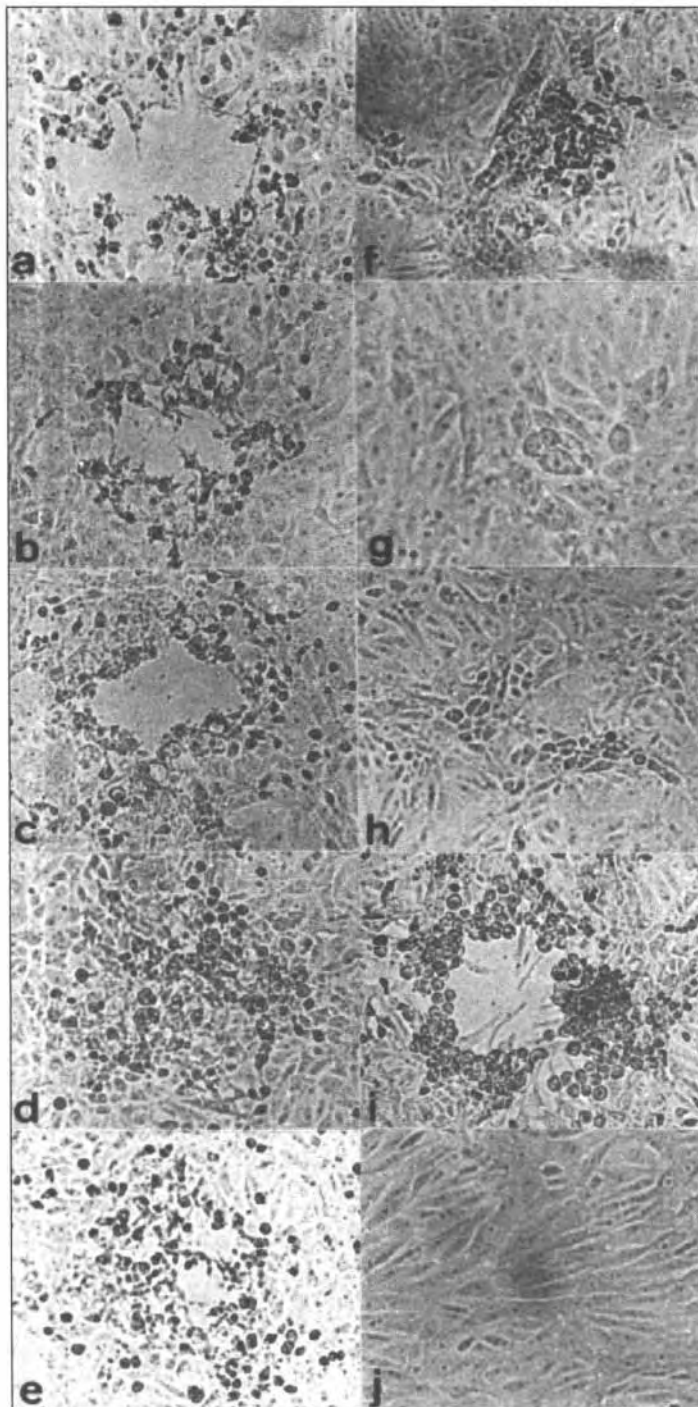


FIG. 1: Formation of viral plaques in Vero cell cultures treated with inocula derived from specimens of patients from the fatal outbreak of childhood viral infection in Sarawak. Representative viral plaques of samples treated with inocula of patients SKV2b (a), SKV5 (b), SKV6 (c), SKVS (d), SKV9 (e), and SKV11 (f) are shown. Cells treated with inocula of patients SKV3b (g) and SKV4b (h) did not show formation of any viral plaques. The viral plaques observed in a - f, showed the formation of what seems to be two merged foci in contrast to that of the herpes simplex virus 1 plaque (i). Mock-treated cells prepared in parallel showed no cellular morphological changes (j). Cells were photographed under 20X objective lens of the Zeiss Televal inverted microscope using the Nikon F3 SLR camera.

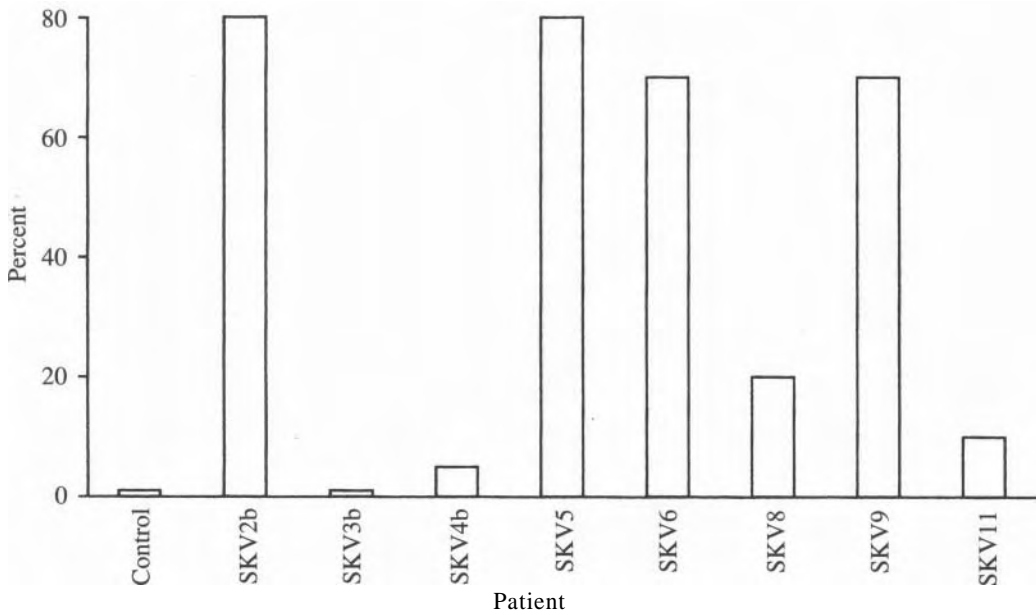


FIG. 2: Percentage of cells manifesting cytopathic effects in Vero cell cultures treated with inocula derived from specimens of patients from the outbreak of fatal childhood viral infection in Sarawak. The percentage of cells manifesting the cytopathic effects were determined by estimating the area on the tissue culture flasks in which the cell monolayer has detached over the total surface area of the tissue culture flask.

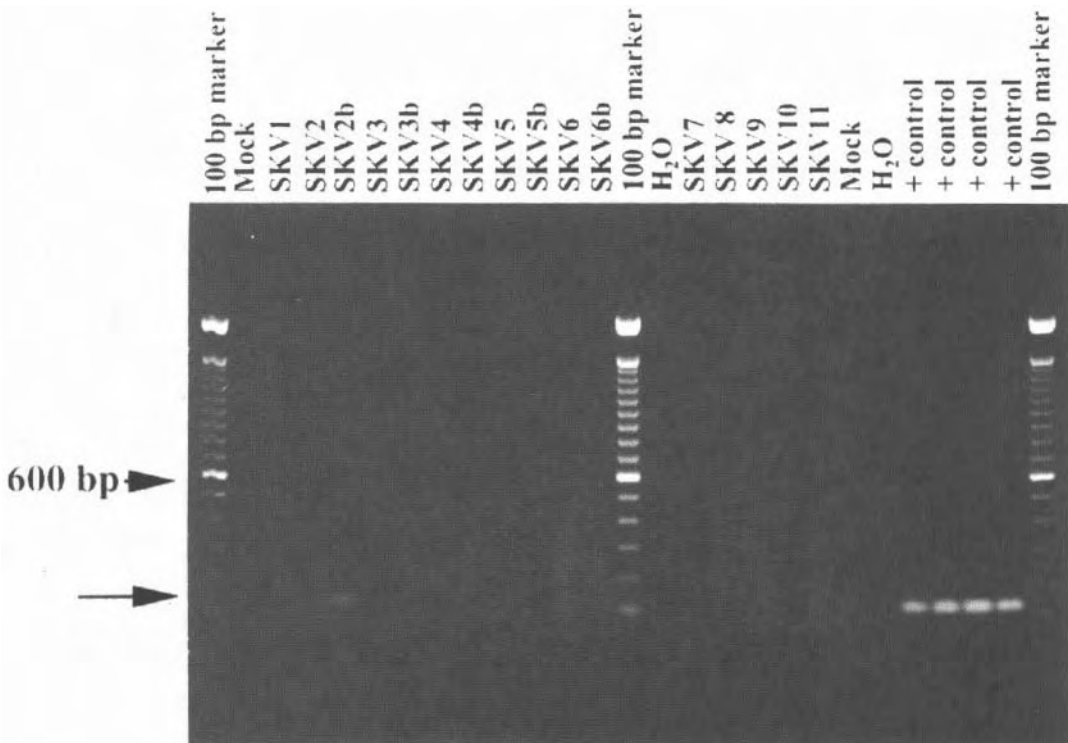


FIG. 3: RT-PCR amplification of patients' samples using pan-enterovirus PCR primers. Amplified DNA was electrophoresed in a 1.5% agarose gel in 0.5X TAE and stained with ethidium bromide. 100 bp DNA ladder was used for size indication. Arrow head indicates the amplified DNA fragment of about 120 bp.

	475	500	520
EV71MS	CCTAACTGCGGAGCACATGCCTTCAACCCAGAGGGTAGTGTGTCGTAATG		
EV71BrCr	CCTAACTGCGGAGCACATAACCCITAATCCAAAGGGCAGTGTGTCGTAACG		
SKV2b	CCTAACTGCGGAGCACATGCCCTCAACCCAAAGGGTATTGTGTCGTAACG *****		
	540	560	
EV71MS	GGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCTT		
EV71BrCr	GGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCTT		
SKV2b	GGCAACTCTGCAGCGGAACCGACTACMTGGGTGTCCGTGTTCCGA *****		

*nucleotide homology.
numbers indicate the nucleotide position.

FIG. 4: DNA sequence comparison between the two reported Enterovirus 71 strains and SKV2b.

encephalitis) antigens using specific monoclonal antibodies. All the antibodies used, however, failed to detect the presence of these viruses' antigens (data not shown). Subsequently, **RT-PCR** performed using total RNA of the infected cells and specific primers for flaviviruses and enteroviruses also failed to amplify any of the viruses genomes except in one patient's sample, **SKV2b**, where a -120 bp DNA product was obtained using the pan-enterovirus primers specific for the enterovirus 5' **untranslated** region (UTR) (Figure 3). No amplification, however, was detectable with any of the pan-enterovirus primer sets^{12,13,14} in the remaining patients' samples, even though the presence of the sequence could be detected in similarly prepared but unrelated **HFMD** samples used as positive controls. DNA sequencing of the amplicon confirmed the presence of EV71 5' UTR **genomic** sequence in patient **SKV2b** (Figure 4). The percentage of nucleotide identity between **SKV2b** isolate and **EV71MS** (ACC # **U22522**) and **EV71BrCr** (ACC # **U22521**) was 94.7% and 93.6%, respectively. This result nonetheless, did not provide convincing evidence for the overall involvement of an enterovirus in the outbreak since no amplification was obtained from the remaining patients.

Separation of total infected cells DNA using pulsed field gel electrophoresis (PFGE) also failed to reveal the presence of any viral DNA. However, DNA smearing was observed in the inocula-treated samples, suggesting that perhaps

the virus presence in the inocula induces cellular DNA fragmentation. DNA fragments ranging in sizes from -2.0 kb to >200 kb were noted in all the patients' samples except patients' **SKV3b** and **SKV4b** samples (Figure 5). Slightly intense EtBr-staining materials of about 9 to 15 kb were noted in patients' **SKV5**, **SKV6** (and **6b**), **SKV8**, **SKV9**, **SKV10**, and **SKV11** samples. Further electrophoresis of these samples, however, did not indicate the presence of any specific large size DNA fragments (data not shown). Rather, a continuous smearing of EtBr-stained DNA fragments comparable to that shown in Figure 5 were noted. This suggests that DNA degradation occurs randomly and did not begin with digestion of cellular genomes into large DNA fragments of any particular sizes. Possible oligonucleosomal cellular DNA fragmentation was then examined. Results obtained from this investigation suggested that oligonucleosomal DNA ladders characteristic in apoptotic cells were distinctly present in all the inocula-treated Vero cells except in those treated with samples of patients **SKV3b** and **SKV4b** (Figure 6). Whereas, **SKV8** and **SKV11** patients' samples which manifested only 20 and 10% cytopathic effects respectively, showed only faint presence of the oligonucleosomal DNA ladders. Similar oligonucleosomal DNA ladders were also seen in dengue 2 virus infected Vero cells DNA prepared 8 days post-infection (data not shown).'' Since equal amount of DNA was used, the absence of oligonucleosomal DNA ladders in

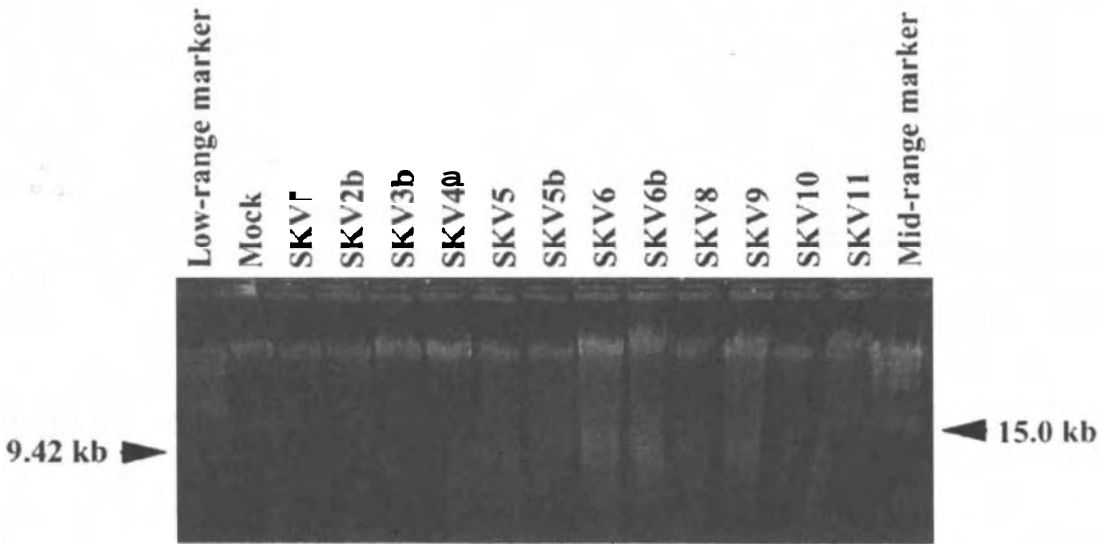


FIG. 5: Pulsed field gel electrophoresis of inocula-treated Vero cells DNA. DNA embedded in low melting point agarose was electrophoresed in a 1.5% agarose at 200 V, 14°C for 7 h with the switch times ramped from 0.1 to 2.5 sec. Fragmentation of cellular DNA is indicated by the presence of EtBr-stained smearing.

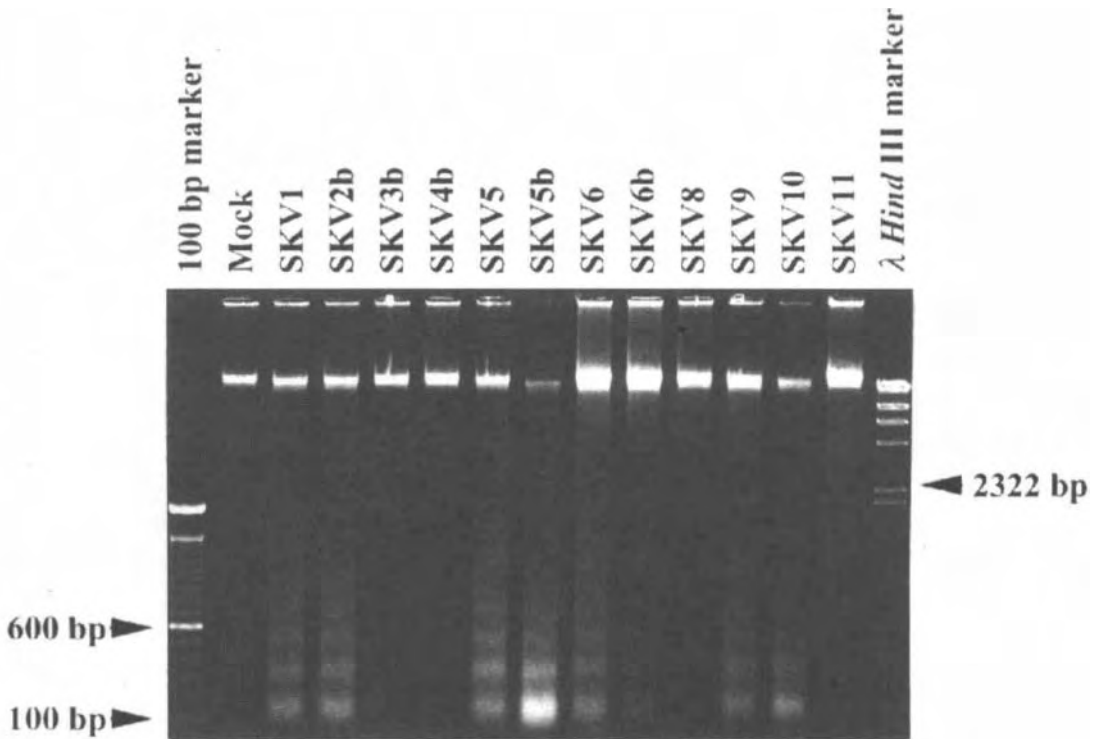


FIG. 6: Detection of oligonucleosomal DNA fragmentation in Vero cells treated with inocula of patients from the fatal outbreak of childhood viral infection in Sarawak. Internucleosomal DNA fragmentation is indicated by the presence of EtBr-stained DNA ladders.

samples of patients **SKV3b** and **SKV4b** could not be attributed to insufficient amount of DNA. The absence, however, corresponded with the absence (< 5%) of cytopathic effects in the infected cell cultures. Staining of the infected cell cultures for the presence of apoptotic cells using TUNEL further revealed that more than 18% of the Vero cells treated with inocula of all patients except **SKV3b** and **SKV4b** were apoptotic (Figure 7). It was observed that by the time when at least 70% of the treated cells have shown cytopathic effects, approximately 30% of the treated cells were apoptotic. Thus, suggesting that induction of apoptosis probably contributes towards the cytopathologic manifestations of the infection in Vero cells.

Induction of apoptotic cellular responses by the patients' inocula was investigated further using the U937 human monocytic cells. Staining for apoptotic cells using TUNEL and extraction of cellular DNA for agarose gel electrophoresis were performed after 6 days PI. **Significant** ($p < 0.01$, T-test) increase in the percentage of apoptotic cells in comparison to the mock-treated control was noted in all inocula-treated samples except for sample treated with patient **SKV4b** inoculum (Figure 7). Cellular oligonucleosomal

DNA fragmentation was detectable by agarose gel electrophoresis of the infected U937 cells in all samples except the mock-treated control (Figure 8). Nonetheless, the **EtBr-stained** DNA ladders were present only faintly even when large amount of extracted cellular DNA was electrophoresed. This result was not surprising since significant DNA ladders were detectable in Vero cells only when at least 20% of the cells have shown cytopathic effects and observable cytopathic effects were not conspicuous if any, in the infected U937 cells. These findings, however, suggest that induction of apoptosis **occurred** also in U937 cells but at a much lower frequency than in Vero cells.

DISCUSSION

In the present report, findings are presented which demonstrate that inocula derived from the clinical specimens of the Sarawak fatal childhood viral infection patients induce apoptotic cellular responses in African green monkey kidney cells (Vero) and to some extent the U937 human monocytic cells. The percentages of apoptotic cells were higher in instances where the cytopathic manifestations

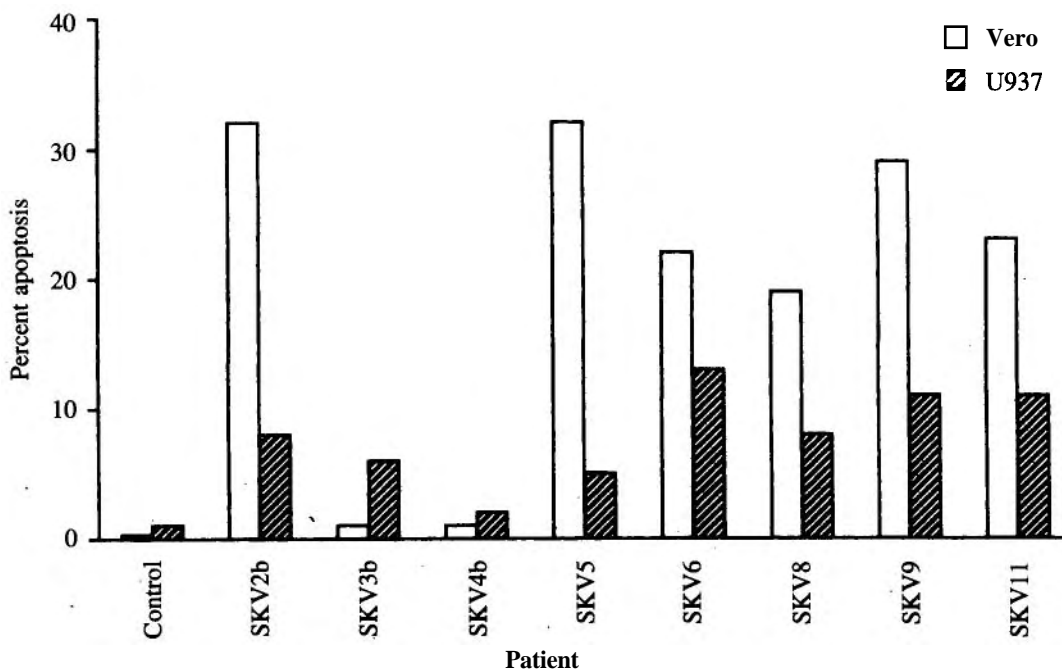


FIG. 7: Frequency of apoptotic cells in Vero and U937 human monocytic cell cultures treated with inocula of patients from the fatal outbreak of childhood viral infection in Sarawak. The percentage of apoptotic cells was determined by counting the number of cells stained fluorescent green with TUNEL over the total number of cells per microscopic field. At least ten microscopic fields were examined per datum.

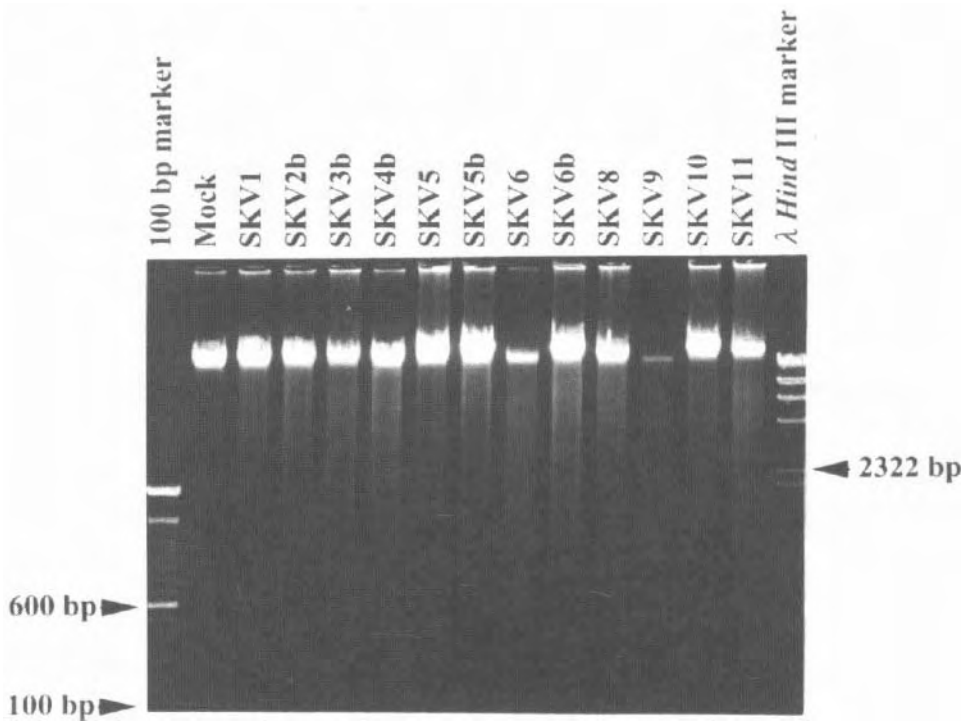


FIG. 8: Agarose gel electrophoresis of inocula-treated U937 human monocytic cells DNA. Faint presence of DNA fragments and DNA smearing were noted in all patients' samples but not in the mock-treated controls.

were also extensive, suggesting that induction of apoptosis could be the mechanism whereby the virus affects the host cells. A number of viruses^{15,16} including dengue, HIV-1¹⁷ and influenza virus,^{18,19} are known to induce cells to become apoptotic. Specific viral proteins such as the adenovirus E1A²⁰ and HIV env and tat²¹, have been shown to induce apoptosis. Since apoptotic cells undergo substantial intracellular physiological changes which lead to cell death, it is suggested that induction of apoptotic cellular responses could act as a host defence mechanism limiting the spread of virus infection.²² This could perhaps explain the difficulty in continuously propagating viruses from patients' specimens in tissue culture cells; as the virus could not replicate efficiently in apoptotic cells, hence, as the cells die the virus is also eliminated. This is evident since attempts to propagate the virus further for most samples were unsuccessful and the cytopathic effects described here for the very early passage inocula were no longer detectable in the later passages. Based on results presented here, however, especially with patient SKV2b, it is suggested that some inocula of patients' from the fatal outbreak in Sarawak

contained perhaps more than one infectious agents or virus variants. The presence of EV71 genomic sequences detectable by the RT-PCR suggests the possible association of the virus in the outbreak. However, since in our investigation only one patient's sample was positive, further evaluation will be required. In addition, the inability to detect the presence of EV71 antigen using immunofluorescence staining with specific monoclonal antibodies in the RT-PCR positive sample, suggests a very low level presence of the virus, if any in other samples, which is not consistent with the presence of viral plaques and extensive cytopathic manifestations in the infected cell cultures. It is also unlikely that the presence of the EV71 genome could not be detected by the RT-PCR in other samples if the viral plaques and cytopathic effects are indeed caused by the virus. Nonetheless, it is postulated that further subcultivation of some of these samples in Vero cells would eventually result in isolation of the less virulent and perhaps contaminating virus or variants if any which were present at a very low level in the initial inocula, at the expense of the apoptotic inducing virulent virus. This is perhaps true since EV71

was eventually isolated, identified and partially sequenced after 4 passages in Vero cells from the initial inoculum of the sample **SKV2b**.² Propagation of the inocula in other cell lines, on the other hand, could result in conditions favouring isolation of the virulent virus from the early passage inocula. Unconfined **report**¹⁰ suggesting isolation of a new fastidious adenovirus from most of the patients' samples lend support to the assertion, since it is known that the adenovirus **E1A** gene can trigger cells to undergo apoptosis.²⁰

As the aetiologic **agent(s)** associated with the fatal outbreak in Sarawak is still unknown, the potential involvement of apoptosis in the rapid deterioration of infected patients' vital functions requires further investigation. Apoptosis which has been associated with normal development of tissues, **teratogenesis**,²³ oncogenesis²² and aging²⁴ is described as co-ordinately regulated cellular events culminating in cell death and disposal of cells involved mechanisms different from that of cell **necrosis**.²⁶ *In vivo*, it is noted that cell death through apoptosis does not evoke inflammatory responses, instead apoptotic cells are phagocytosed by neighbouring intact cells, causing minimal damage to the surrounding cells or **tissues**.²⁷ As such, evidences of inflammatory responses and **scarring** of the tissues do not occur. Furthermore, it has been shown that apoptotic cells were detectable in the hearts of patients with **cardiomyopathies**^{28,29,30} and in the brain cortex of patients with spongiform **encephalopathy**.³¹ In addition, induction of apoptosis directly affecting *in vivo* tissues by viruses have also been demonstrated with HTLV **I**³², **HIV-1**³³, **Reovirus**³⁴ and **Coxsackievirus infections**³⁵. Thus, it is possible that induction of apoptosis in either the brain or heart of patients from the fatal outbreak of childhood viral infection in Sarawak could contribute towards the disease and yet the damage is not detectable unless specific apoptotic staining is performed on the tissues.

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