Infectious Haematopoietic Necrosis Virus-Induced Cell Death in Fish Cell Culture

A. GREGORC [1*], I. D. BOWEN[2], V. JENČIČ[1], P. HOSTNIK[1]
[1]Veterinary Faculty of the University of Ljubljana, Ljubljana, Slovenia
[2]Cardiff School of Biosciences, Biomedical Building, Cardiff University, PO Box 911, Cardiff CF10 3US, U.K.

Received June 7, 2004
Accepted March 3, 2005

Abstract

Gregorc A., I. D. Bowen, V. Jenčič, P. Hostnik: Infectious Haematopoietic Necrosis Virus-Induced Cell Death in Fish Cell Culture. Acta Vet. Brno 2005, 74: 279-285.

Cell death development was investigated in the epithelioma papulosum cyprini (EPC) cell line after infection with infectious haematopoietic necrosis virus (IHNV). Cytopathic effect (CPE) was evaluated, immunohistochemistry for virus localisation and the development of cell death were studied. Modes of cell death were characterised by staining with TdT-mediated dUTP nick end labelling (TUNEL), and with Annexin V and propidium iodide.

CPE was evaluated as 25%, 50%, 75% and 100% at the virus culture infective concentration doses (TCID50/0.1 ml) of 1.25, 6.25, 31.25 and 156.25, respectively, and no CPE was found at the concentration lower than 0.25 TCID50/0.1ml. At the PBS:virus concentration 0.25 TCID50/0.1 ml only a few cells were found anti IHNV antibody positive, and no CPE was observed. In the range of virus concentrations from 6.25 to 156.25 TCID50/0.1 ml all cells were positive. The level of positive cells using the TUNEL technique increased 30% and 55% after the increasing the CPE up to 50% and 75%, respectively. At the higher levels of CPE, all the remaining cells were positive.

Infected cells exhibited features of early stage of apoptosis as demonstrated reactivity with annexin V. Apoptotic cells expose external phosphatidylserine residues while preserving membrane integrity. Red fluorescence with propidium iodide is associated with increased levels of necrosis and appeared in cells simultaneously with FITC marker and also alone in vacuolated and enlarged necrotic cells. Apoptosis appears before the pathological changes of necrosis and it is correlated to the spread of infection. The relevance of the current view that IHNV infection includes at least two modes of cell death: apoptosis and necrosis, is discussed.

Epithelioma papulosum cyprini, cell death, necrosis, immunohistochemistry, annexin V, TUNEL

Cellular death can occur either by accident, referred to as necrosis, or by design variously described as physiological cell death, programmed cell death or apoptosis (Bowen et al. 1996). Apoptosis presents a range of morphological symptoms including cell shrinkage, chromatin margination followed by DNA fragmentation and a florid break-up of the cell into spherical apoptotic bodies (Wyllie 1981). It has been shown that apoptosis can be induced by genetic means (Ellis et al. 1991), but can also be induced by non-genetic means (Arends and Wyllie 1991). Necrotic cell death appears to be induced under extreme conditions such as ischaemia, hypoxia, toxin, hyperthermia. The subsequent morphological changes encompass oedema following calcium overload, dilatation of the endoplasmic reticulum, polysome disaggregation, and mitochondrial swelling. The chromatin condenses, and clumps are formed at the nuclear periphery (Bowen et al. 1996). Necrosis refers to the *post mortem* changes that occur following the death of the cell (Trump and Berezesky 1998).

A range of approaches are available to demonstrate the apoptosis of cells in living tissue or cell suspensions. Immunocytochemical methods assaying DNA fragmentation (Trauth 1995) and Annexin V (Martin et al. 1995) for the localisation of exposed phosphatidylserine; are useful techniques for detecting impending programmed cell death. DNA breakdown preceding nuclear collapse of apoptotic nuclei can be tested using the

Address for correspondence:
Prof. Dr. Ales Gregorc
Agricultural Institute of Slovenia
Hacquetova 17, SI-1000 LJUBLJANA

Phone:+386 (0)1 280 51 50
Fax: +386 (0)1 280 52 55
e-mail: ales.gregorc@kis.si
http://www.vfu.cz/acta-vet/actavet.htm

terminal deoxynucleotidyl transferase mediated dUDP nick end labelling, termed TUNEL assay (Levy et al. 1998; Sgonc and Gruber 1998).

The causative agent, infectious hematopoietic necrosis virus (IHNV) belongs to the *Rhabdoviridae* family, the new genus *Novirhabdovirus*, enveloped bullet-shaped virus with single molecule of linear, negative sense ssRNA, containing genes for structural proteins. It consists of a helical nucleocapsid surrounded by a viral membrane composed of host lipids and proteins (Murphy 1995).

After adsorption, the IHNV penetrates into the host cell cytoplasm. The virions aggregate in large endosomes. The viral membranes fuse to the endosomal membranes and the complex binds with the glycoprotein, and the completed virus buds from the plasma membrane (Schütze 1995).

Cell death was previously characterised in our laboratory using terminal deoxynucleotidyl transferase (TdT)-mediated biotin-dUTP nick end labelling (TUNEL) in honey bee midgut (Gregorc and Bowen 2000) and Annexin V in salivary gland of honeybee larvae (Silva de Moraes and Bowen 2000). A series of approaches to detect cell death were conducted in different cultured cells using TUNEL (Wutzler et al. 2001) and Annexin V or both techniques (Bell et al. 2001; Buemi et al. 2001). Apoptosis was evaluated in viral haemorrhagic septicaemia (VHS) and infectious pancreatic necrosis (IPN) infected rainbow trout *in situ* (Eléouët et al. 2001). In this study the induced cell death was investigated in fish cell culture after IHNV infection. Immunoperoxidase virus labelling, Annexin V-FITC with propidium iodide (PI) fluorescence and apoptosis TUNEL assays have been employed in an attempt to define the modes of cell death and for the broad quantification of cell death observed in IHNV infected fish cell culture.

Materials and Methods

Preparing cell cultures and in virus infection

The epithelioma papulosum cyprini (EPC) cell line (286 passage) was kindly supplied by dr. G. Bovo from the Instituto Zooprofilatico Sperimentale, Padova, Italy and propagated routinely at 25 °C (Fijan et al. 1983) in Eagle's minimal essential medium (MEM, Gibco BRL, Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum and antibiotic Garamycin (Lek d.d., Ljubljana).

Infectious haematopoietic necrosis virus (IHNV), first French isolate (Hattenberger-Baudouy et al. 1987) from Rainbow trout adapted to EPC cell culture was obtained from the Inter-laboratory Proficiency test for the National reference laboratories for fish diseases in 1998. IHNV stock prepared in EPC cells, had a titer of $10^{5.3}$ tissue culture 50% infective doses in 0.1 ml (TCID50/0.1 ml) as calculated by Reed and Muench (1938).

Five-fold dilutions (2.0 ml cell culture medium in 10% fetal bovine serum (FBS) and 0.5 ml of sample) of IHNV isolate were performed. Each dilution (100 µl) in the range from 1:50 to 1:105.7 was transferred on EPC cell monolayers growing on coverslips in 24-well plates. After adsorbtion of the virus for 30 min at 15 °C, 2 ml of MEM in 10 % FBS per well was added. Microplates were then incubated in 5% CO_2 at 15 °C and CPE was observed microscopically.

Measuring the cytopathic effect (CPE)

Confluent infected EPC cells monolayers on coverslips were observed for the effect of virus that could be manifest by cell degeneration or cytopathic effect (CPE) (Grist et al. 1979) and compared to uninfected cells. Observations of native unstained cultures were performed daily using Zeiss inverted light microscope. CPE is a routine laboratory method defined as a focal area of lysed cell monolayer on coverslips (Wolf 1988; Landry and Hsiung 2000). These areas are compared to the areas covered by cultivated control, uninfected cell monolayer, evaluated and expressed in percentage. The degree of lysed monolayer is determined as low, approximately 25% lysed area in a well, middle level lysis as 50%, high level lysis 75% and the total lysed area as 100% as a standard method to evaluate CPE (Landry and Hsiung 1986). The percentage of lysis was estimated by observing the whole cell monolayer compared with the cell monolayer in control wells. Each virus dilution was applied to cell monolayers in four replicates and then microscopically examined.

Immunoperoxidase labeling

EPC cells cultivated on coverslips were IHNV infected with the indicated tissue culture infective doses (TCID50/0.1ml) and incubated in 5% CO_2 for 15 min at 15 °C. The cell culture medium was removed and coverslips with adherent cells were air–dried and fixed in ice cold acetone 85% for 30 min and washed in phosphate buffer solution, containing 5% Tween 20 (PBS-T) pH 7.2.

Endogenous peroxidase was blocked with PBS/hydrogen peroxide (1%) mixture for 30 min at room temperature.

After washing in PBS-T (Phosphate buffer saline containing 0.05% Tween 20, pH 7.2), monoclonal anti-IHNV (Bio-X Diagnostics - 34, Rue de Luxembourg, Belgium) antibody diluted in PBS-T (1:40) was applied and then incubated with adherent cells for 60 min at 37 °C in a humidified chamber. Cells were rinsed with PBS-T and rabbit-anti mouse peroxidase-conjugated antibody (Dako P260, Denmark) was applied and incubated for 60 min at 37 °C. The substrate 3-amino-9-ethylcarbazole (AEC, Sigma, A-6926, St. Louis, Missouri, USA) was used. One tablet of AEC was dissolved in 5 ml of Dimethylformamide (DMF, Sigma Product D-4254, St. Louis, Missouri) and 2.5 ml of AEC solution containing 25 µl 30% H_2O_2 was added. Cells on coverslips were incubated in substrate solution for 15 min at room temperature and rinsed in distilled water. Four replications were made for each virus dilution. As a control, no primary antibody was used and endogenous peroxidase activity was quenched. In the second control no primary antibody was used and endogenous peroxidase was not quenched. The percentage of immunoperoxidase stained cultured cells was established by counting at least 100 cells per microscopic field and recording those showing the DAB labelling. Light microscopy and photography were conducted using Nikon light microscope.

TUNEL technique, using "In situ cell death detection kit" (Roche) (ISCDDK)

Air dried infected and uninfected cell cultures were incubated with paraformaldehyde solution in PBS (4%) at pH 7,4. for 30 min at room temperature. Slides were rinsed with PBS and incubated with proteinase K (20 µg/ml in 10 mM Tris/HCl, pH 7.4), for 15 min in an incubator at 37 °C. Labelling was conducted by covering the tissue section with approximately 30 µl 'tunel reaction mixture' composed of terminal deoxynucleotidyl transferase (TdT) from calf thymus, for 60 min at 37 °C in a humidified chamber. TdT enzyme incorporated fluorescein was detected with "converter-AP" consisting of anti-fluorescein antibody from sheep, conjugated with alkaline phosphatase (included in the kit). Naphthol ASTR phosphate was used as a substrate for the phospahatase reporter enzyme and Fast red violet (both from Sigma, St. Louis, USA) as a coupler. The medium operated at pH 8.4. Counterstaining was accomplished by transferring into methyl green for 10 min at room temperature, washed in distilled water and mounted in aqueous mounting medium. Four replications were carried out for each virus dilution, counting more than 200 cells and recording red azo-dye staining localised to the nuclei or cell cytoplasm. As a negative control, labeling was conducted with label solution without terminal transferase.

Annexin test, using Annexin V-FITC apoptosis detection kit (Sigma)

The method provided by Sigma protocol for resuspended cells was modified for air dried infected and uninfected cell cultures placed on to slides. Cells were washed in PBS and fixed with paraformaldehyde solution in PBS (4%) at pH 7,4 for 15 minutes at room temperature. After washing in PBS, approximately 30 µl of 1 X binding buffer was applied to the cells for 15 min at room temperature. Binding buffer was removed by vacuum pump and cells were incubated with solution of AnnexinV-FTC (5 µl) and propidium iodide (10 µl) in 1 X binding buffer (500 µl) for ten min in the dark at room temperature. After incubation, slides were gently dried using vacuum pump, mounted in aqueous medium and covered with glass coverslips. The cells were observed with a Nikon fluorescence microscope using filter set for FITC and rhodamine.

Results

Cytopathic effects (CPE) and immunoperoxidase (IP) staining

Six days after EPC cell monolayers infection, the CPE were evaluated microscopically for the level of cell lysis on coverslips of four replications. Approximate degree of CPE of the cell monolayer evaluated as 25%, 50%, 75% and 100% was found at the virus concentration of 1.25, 6.25, 31.25 and 156.25 respectively (Plate XV, Fig. 1A). The virus concentration was calculated previously according to the Reed and Muench (1938) method. No CPE were found at the concentration lower than 0.25 $TCID_{50}$/0.1ml. In this initial stage of infection brown reaction product was found localised in separate clusters. Some cells were swollen with evident pycnotic nuclei and condensed cells were also present (Plate XV, Fig. 1B).

Anti-IHN virus antibody carries a peroxidase conjugated reporter enzyme and the DAB substrate results in a brown staining. Brown reaction product is indicative for virus localisation in the cell cytoplasm. The cell culture destruction expressed in the level of CPE and in the number of IP stained cells were counted. At the virus concentration of 0.05 $TCID_{50}$/0.1 ml no CPE and IP were found in all replications (Table 1). The difference of DAB localisation and level of CPE depended on the virus dilution (concentration) applied to the culture. At the virus:PBS concentration 0.25 $TCID_{50}$/0.1 ml only a few DAB positive cell were found in three out of four culture monolayers, and no CPE was observed. DAB reaction product (Fig. 1B) localizes the site of antibody attachment. In the range of virus

concentrations from 156.25 $TCID_{50}$/0.1 ml to 6.25 $TCID_{50}$/0.1 ml all cells in four replications showed DAB reaction product. A characteristic increase of DAB reaction product was found in cells present in virus treated culture, thus demonstrating a positive dose-dependent correlation (Plate XV, Fig. 1C).

Table 1. Comparative effects of different dilutions of infectious hematopoietic necrosis virus applied on EPC. Evaluations were carried out on the cytopathic effect (CPE), virus localisation using Immunoperoxidase labeling (IP), and apoptosis detection using *In situ* cell death detection kit (ISCDDK). Analyses were conducted 6 days after infection, data were expressed as percentage of positive cells, ± SD is given for IP and ISCDDK.

IHNV contained (TCID 50/0.1 ml)	CPE (%)	IP (%)	ISCDDK (%)
156.25	100	100	100
31.25	75		55 ± 20.81
6.25	50		30 ± 8.16
1.25	25	87.5 ±1 4.43	13.7 ± 2.50
0.25		sporadic cell clusters	Sporadic cells
< 0.05	0		Sporadic cells
uninfected - control	0	0	

ISCDDK

DNA strand breaks labelled with fluorescein following application of anti-fluorescein antibody conjugated with alkaline phosphatase, as advised in the Roche *'In situ* cell death detection kit, AP' (ISCDDK) identified red azo-dye reaction product in nuclei of dying cultured cells.

In uninfected cultured cells, reaction product was found in sporadic cells at a level lower than 0,1% (Plate XVI, Fig. 2A). Uninfected cells in those cell monolayers were preserved and no CPE was observed.

In infected cultured cells reaction product was found distributed in the nuclei and in the cell cytoplasm. A positive reaction in cells increased with the virus concentration. The level of positive cells characteristic for cell death using ISCDDK was determined as 1% and CPE was not observed (Fig. 2B). When approximately 25 % cultured cell were lysed, approximately 15% cell were found ISCDDK positive. The level of identified ISCDDK positive cells increased on the level of the order of 30% and 55% after the increasing the CPE up to 50% and 75%. (Fig. 2C).

At the higher level of CPE when the majority of cultured cells are lysed, the remaining spherical cells were positive (Plate XVI, Fig. 2D). Positive cells containing red azo-dye reaction product showed an increase in vacuolation. Controls omitting TdT enzyme, displayed no red reaction product.

Annexin V-FITC and propidium iodide assay

V-FITC is indicative for an early symptom of apoptosis. Apoptotic cells expose external phosphatidylserine residues while preserving membrane integrity. Both infected and uninfected cultured EPC exhibited alterations characteristic of apoptosis, Annexin V staining and morphological changes such are cell condensation and shrinkage (Fig. 3A). All infected cultures contained apoptotic cells, but in control uninfected cultures apoptotic cells were rare.

In infected cultured cells there appeared to be an increase of phosphatidylserine expression in apparently prenecrotic and postnecrotic cells demonstrated by Annexin V-FITC and propidium iodide labelling. Some cells showed internalised FITC marker (Plate XVII, Fig. 3B). Red fluorescence with propidium iodide indicative for lost membrane integrity associated with necrosis appeared in cells simultaneously with FITC marker and

also alone in vacuolated and enlarged necrotic cells (Fig. 3C). Only FITC and propidium labelled positive cells were microscopically recorded.

Discussion

In adherent infected EPC line INHV was localised, using monoclonal anti-IHNV antibody. At the lowest infective dosage, six days after infection two types of morphological changes were found. Swelling, increasing the cell volume with cell disintegration proceeding to complete dissolution typical for cell necrosis and condensation of nuclei and cytoplasm typical for apoptosis (Wyllie 1981). Virus localisation in these condensed cell compartments may be indicative of virus induction specific apoptotic morphological changes. Increasing infective virus concentration induces both main morphological characters, cell swelling and nuclei condensation and CPE is increased up to 100%. In control uninfected cells only sporadic vacuolated and condensed cells characteristic for necrosis and apoptosis were found. This seems to be indicative of a normal level of cell death in uninfected cell monolayer.

Higher levels of cell death were found with increasing virus concentration and expressed CPE. At the IHNV concentration 31.25 TCID50/0.1 ml and 6.25 TCID50/0.1 ml approximately 50% apoptotic cell death was found and CPE was estimated between 50 and 75%. ISCDDK showed induction of DNA strand breaks at 13% with the CPE of the order of 25%. Apoptosis is also induced in cultured cells after bovine respiratory syncytial virus infection (Cristina et al. 2001) and in rainbow trout tissues after viral haemorrhagic septicaemia and infectious pancreatic necrosis virus using TUNEL technique (Eleouët et al. 2001). Apoptotic cells were found mainly peripherally of the preserved cells surrounded by a field of lysed cells. In control uninfected cultures only sporadic apoptotic cells were found indicative of a normal levels of cell death.

Loss of phospholipid asymmetry is indicative of apoptotic cells, which bind with Annexin–V. Necrosis is separately detected using propidium iodide uptake. The Annexin–V technique (Martin et al. 1995) demonstrated an early stage of apoptosis in INHV infected EPC cultured cells. Sporadic binding occurs in uninfected cell culture and in progressive CPE induced by INHV infection. Parallel uptake of propidium iodide in the latter case demonstrated an incipient level of secondary necrosis. At this stage CPE is evaluated as 50%.

In EPC cultured cells INHV induces both apoptosis and necrosis. Apoptotic cells were found in a fish cell line infected with infectious pancreatic necrosis virus (IPNV) assayed with TUNEL technique and Annexin–V FITC labelling (Hong et al. 1998). It seems that apoptosis appears before the pathological changes of necrosis and it is correlated to the spread of infection in cultured cells and to the evaluated CPE. At least two modes of cell death were observed. We conclude that IHNV infected EPC cells die by apoptosis and necrosis and that the TUNEL technique and Annexin V staining demonstrated the pattern of apoptotic cell death that usually precedes necrotic cell deletion.

Annexin–V FITC positive fluorescence indicative of phosphatidylserine externalisation as a symptom of early apoptosis is clearly seen after INHV infection. The ISCDDK method shows induction of DNA strand breaks occurring in apoptosis and propidium iodide uptake denotes degraded cell membrane. A difference was found between the ISCDDK results and the uptake of propidium iodide and Annexin test. This suggests that there may have been only a background level of early apoptosis-positive product in these cells as demonstrated by the Annexin–V assay. In IHNV infected EPC cells ISCDDK appears to show apoptotic and necrotically induced changes similar to those found in bee larvae (Gregorc and Bowen 2000; Silva de Moraes and Bowen 2000). IHNV infection does appear to cause increased apoptosis as was found in fish cell line infected with infectious pancreatic necrosis

virus (IPNV) (Hong et al. 1998) and precedes necrosis as was assayed by the ISCDDK and propidium iodide.

Virus infekční nekrózy hematopoetické tkáně vyvolávající buněčnou smrt v buněčné kultuře ryb

Byl zkoumán vývoj buněčné smrti na buněčné linii *epithelioma papillosum cyprini* (EPC) po infekci virem infekční nekrózy hematopoetické tkáně (IHNV). Byl hodnocen cytopatický efekt (CPE), dále byla studována lokalizace viru pomocí imunohistochemických metod a vývoj buněčné smrti. Způsoby buněčné smrti byly charakterizovány metodou TUNEL, pomocí barviv Annexin V a propidiumjodid. Při koncentracích 1.25, 6.25, 31.25, resp. 156.25 infekčních dávek (TCID50) ve virové kultuře byl hodnocen CPE jako 25 %, 50 %, 75 % a 100 %, při koncentraci nižší než 0.25 TCID50 nebyl CPE nalezen. V PBS při koncentraci viru 0.25 TCID50 pouze několik buněk vykazovalo pozitivitu na přítomnost IHNV protilátek a cytopatický efekt nebyl pozorován. V rozmezí virových koncentrací od 6.25 do 156.25 TCID50 byly všechny buňky pozitivní. Stupeň pozitivity buňek se při používání TUNEL techniky zvýšil o 30 % a po té, co se zvýšil cytopatický efekt o více než 50 %, respektive 75%, se stupeň pozitivity zvýšil o 55 %. Při vyšších stupních CPE byly všechny zbylé buňky pozitivní. Infikované buňky vykazovaly rysy raného stádia apoptózy jako důkaz reaktivity s Annexinem V. Apoptické buňky exponují vnější fosfatidylserinová residua, zatímco si zachovávají membránovou integritu. Červená fluorescence po obarvení buněk propidiumjodidem je spojena se zvyšujícími se stupni nekrózy spolu se simultánním objevením FITC markeru v buňkách, ale také může být pozorována samostatně ve vakuolizovaných a zvětšených buňkách. Apoptóza se objevuje před patologickými změnami nekrózy, a tím koreluje s rozšířením infekce. Závažnost tohoto aktuálního pohledu, že IHNV infekce zahrnuje přinejmenším dva způsoby buněčné smrti: apoptózu a nekrózu, je diskutována.

Acknowledgements

Financial support for the research project conducted by research group No. P4-0092 and P4-0072 of the Slovenian Ministry of Education, Science and Sport and the financial support of The Royal Society (UK) and Slovenian Academy of Science are gratefully acknowledged.

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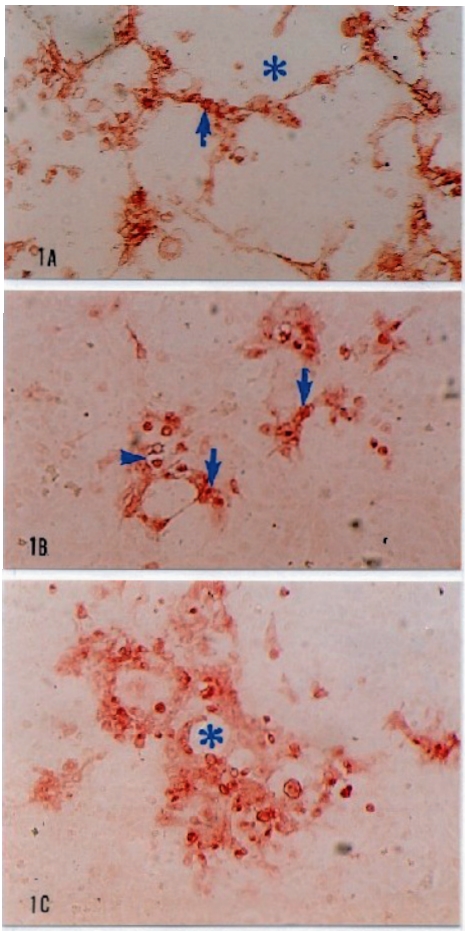

Fig. 1. The epithelioma papulosum cyprini (EPC) cells six days after IHNV infection fixed in 85% ice cold acetone, exposed to the monoclonal anti-IHNV antibody (Bio-X, Denmark). Panel A: The staining is localised to the remaining cells (▲) between areas of lysed cells (✱). CPE was estimated as 100%.
Panel B: The cultured cells received 0.25 TCID50/0.1 ml virus concentration. DAB reaction product is localised to the sporadic infected cells. Note the absence of DAB reaction product in cytoplasm of some swollen cells (►) and cell shrinking and condensation with intense reaction product (▲). No CPE was observed.
Panel C: detectable localisation of IHNV in separate zones of the cultured cells. Initial stage of CPE is observed (✱) after viral infection using dilution 1:31.250. Magnification × 100

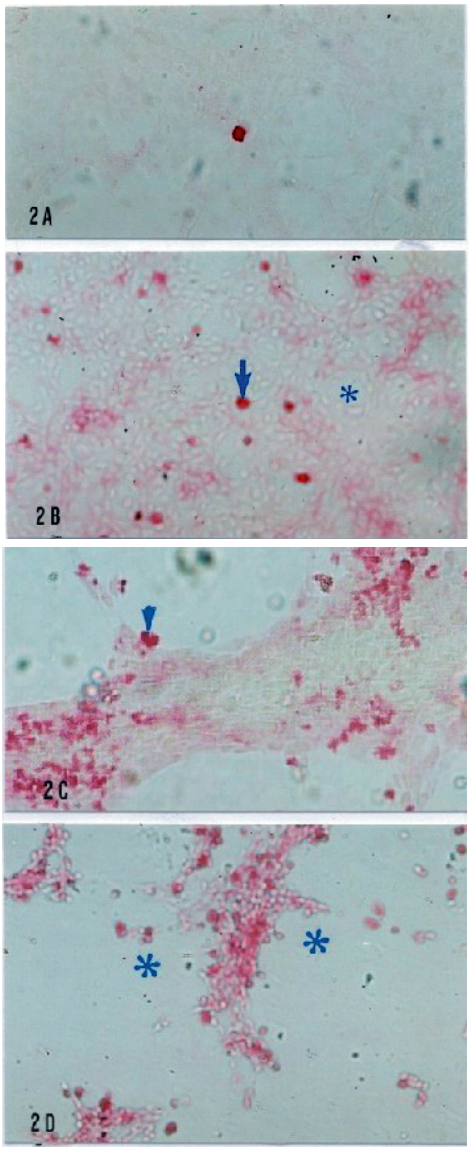

Fig. 2. The epithelioma papulosum cyprini (EPC) cells six days after IHNV infection fixed in paraformaldehyde. Cell death was detected by the TUNEL technique using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP for DNA labelling, an anti-fluorescein alkaline phosphatase conjugated antibody (ISCDDK), fast red for visualization, and counterstaining with methyl green. Panel A shows dens red azo-dye staining localised to the sporadic cells, in the level of less than 0.1% in not infected (EPC) cells. Panel B shows sporadic dens red azo-dye staining (↟) of the IHNV infected cultured cells with low level of CPE (✱). Panel C shows increased dens red azo-dye staining of the infected cells localised in the peripheral area of preserved cells (►). Panel D shows dens red azo-dye staining localised to the cells still persisting in the field surrounded by lysed areas (✱). Approximately 75% CPE is observed. Magnification × 100

Plate XVII

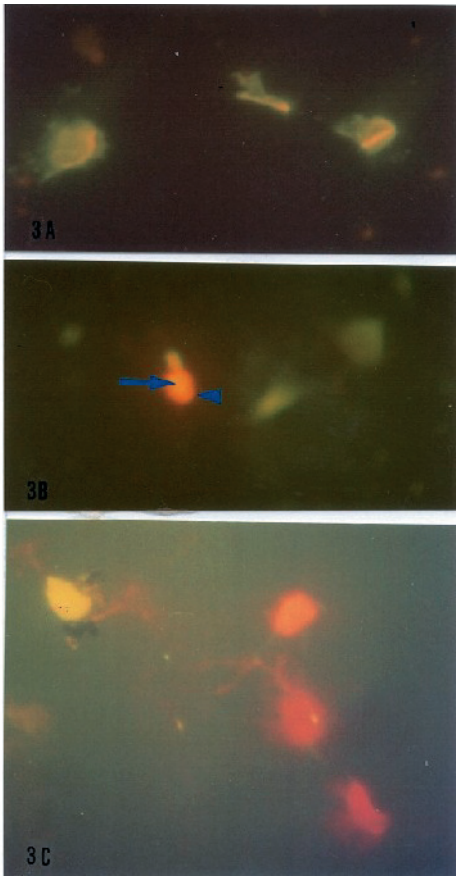

Fig. 3. Annexin V-FITC positive fluorescence indicative for phosphatidylserine externalisation, an early symptom of apoptosis and entry of propidium iodide as a symptom of necrosis in IHNV infected EPC cells. Panel A shows phosphatidylserine externalisation of the order of 1% in infected cell culture. Cells show shrinkage and the outer cell membrane is positive. Panel B shows the internalisation of Annexin (▲) and red fluorescence of the cytoplasm confirming entry of propidium iodide (►) and loss of membrane integrity. Panel C shows cells with both Annexin for apoptosis and red fluorescence indicative of secondary necrosis in IHNV infected cultured cells. Magnification × 400