Apoptosis induced by infectious bursal disease virus

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Chicken peripheral blood lymphocytes (PBLs) show morphological and biochemical features of apoptosis (programmed cell death) when infected in vitro with infectious bursal disease virus (IBDV). DNA extracted from IBDV-infected lymphocytes displayed an intense laddering pattern when visualized after agarose gel electrophoresis. IBDV-infected PBLs had significantly higher apoptotic and necrotic indices measured by acridine orange–ethidium bromide staining than did control lymphocytes. Electron micrographs of the IBDV-infected PBLs revealed features typical of apoptosis such as peripheral condensation of chromatin, blebbing of the plasma membrane and fragmentation of the nucleus and of the cell leading to the formation of apoptotic bodies. These findings indicate that IBDV, in addition to causing necrosis, can also induce apoptosis in avian lymphocytes in vitro.

Apoptosis is an individual and active type of cell death that is characterized by nuclear fragmentation and cellular breakdown into apoptotic vesicles (Cohen, 1991). Unlike necrosis, there is no release of cellular contents into the interstices and consequently no inflammation surrounding the dead cells (Wyllie, 1981). This type of cellular self-destruction is usually initiated by physiological stimuli, but pathological stimuli, such as viral infections (Groux et al., 1991; Ameison & Capron, 1991; Hügin et al., 1991; McCabe & Orrenius, 1992; Meyaard et al., 1992; Rojko et al., 1992; Jeurissen et al., 1992; Razvi & Welsh, 1993; Galvan et al., 1993), can also be the triggering factor.

Infectious bursal disease virus (IBDV) is a known cause of immunosuppression in chickens; the mechanism of this immunosuppression is not well understood. It has been determined that the virus has a direct cytopathic effect on immature B cells causing severe necrosis, lymphoid depletion and subsequent immunosuppression (Müller, 1986; Burkhardt & Müller, 1987). There are also reports that the infected bursa of Fabricius (BF) undergoes a very rapid and extensive atrophy with little or no inflammatory response (Rosenberger et al., 1985; Sharma et al., 1989; Snyder, 1990; Lukert & Saif, 1991). These chickens, however, are also immunosuppressed. Immunosuppression without severe inflammatory response of the BF is an unexplained phenomenon. In this paper, we examine the possibility that IBDV infection of chicken lymphoid cells may cause apoptosis and lead to immunosuppression.

Five ml of heparinized blood was taken from white leghorn chickens that did not have pre-existing antibodies against IBDV, layered over Histopaque 1083 (Sigma) and was centrifuged at 2000 g for 15 min. Lymphocytes at the interphase were collected and washed three times with RPMI 1640 medium (Gibco). The cells were counted and a population of about 10⁷ cells/ml was divided into three groups. The virus-infected group received 2 ml of IBDV serotype 1 (strain L) which had been propagated and titrated in chick embryo fibroblasts and had a TCID₅₀ of 10⁶. The positive control group received 2 ml of 5 µg/ml hydrocortisone (Sigma). The negative control group received 2 ml of RPMI 1640 medium. All three treatment groups were then incubated at 37 °C for 6 h in a humidified CO₂ incubator.

At 0, 2, 4 and 6 h after the start of incubation, 25 µl of cells from each group was removed and stained with 1 µl of acridine orange (100 µg/ml)–ethidium bromide (100 µg/ml) solution. Ten µl of the stained cell suspension was placed on a glass slide and covered with a 22 mm² coverslip. The slides were examined under u.v. illumination (Carl Zeiss). Each sample was counted four times and each time 200 or more cells were counted. The characteristics of the cells were recorded according to the colour and structure of the chromatin (Duke & Cohen, 1992). The mean and standard deviation of the four

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counts of each different cell characteristic were used to calculate the apoptotic, the necrotic and the cell viability indices and their respective error bars. Two-sample Student's t-tests were performed to compare the indices at different collection times, using Minitab Release 9 for Windows (Minitab). No statistical differences were detected between the apoptotic indices at 0 h on all samples. At 2, 4 and 6 h post-treatment, IBDV and hydrocortisone induced higher levels of apoptosis (Fig. 1a) in PBLs than the background apoptosis seen in the negative control (P values < 0.019). The apoptotic features were most intense at 6 h post-treatment. In vitro infection of PBLs with IBDV seems to accelerate and increase apoptotic cell death. The appearance of apoptosis in the negative control cells after 6 h of incubation may be due to the prolonged in vitro incubation of lymphocytes. Even under the best nutrient and temperature conditions in vitro incubation is no substitute for the in vivo environment and may in fact help the activation of programmed cell death. It has been reported that apoptosis is a continuing and physiological process in chicken lymphoid cells and that as many as 5-7% of bursal cells are undergoing apoptosis at a given time (Motyka & Reynolds, 1991; Asakawa et al., 1993).

In terms of necrosis induction, there were no differences among the three groups at 0 h post-treatment. At 2 and 4 h post-treatment, IBDV induced a significantly higher necrotic index than negative and positive controls (P values < 0.047). At 6 h post-treatment, the only statistically significant difference detected was between IBDV-infected cells and the positive control (P < 0.035), and no difference was observed between IBDV-infected cells and the negative control or between the positive and negative controls (Fig. 1b). In these experiments the incidence of necrosis, shown in Fig. 1(b), did not increase as much as apoptosis (Fig. 1a). These findings apparently reinforce the importance of apoptosis as an event in IBDV infection of lymphoid cells.

No differences in cell viability (Fig. 1c) were detected among the three groups at 0 h post-treatment; however at 2 and 4 h post-treatment, IBDV-infected cells showed lower viability than did the negative and positive controls (P < 0.046). At 6 h post-treatment, the IBDV-infected and positive control groups had lower viability indices than the negative controls (P < 0.0010 and < 0.0017, respectively).

IBDV-infected PBLs were also used for DNA fragmentation studies. At 0 h and 6 h after the start of incubation the cells were lysed with TTE (10 mM-Tris–HCl, 0.25% Triton X-100, 1 mM-EDTA). After treatment with phenol–chloroform–isoamyl alcohol (25:24:1), the DNA was precipitated in the presence of 0.3 M-sodium acetate and cold absolute ethanol at −20 °C overnight, and then resuspended in 10 mm-

Fig. 1. Apoptosis (a), necrosis (b) and cell viability (c) in avian lymphocytes as measured by ethidium bromide-acridine orange staining. Symbols: □, negative control; □, IBDV-treated cells; □, hydrocortisone-treated cells.

Fig. 2. Agarose gel electrophoresis of the DNA. There is no DNA fragmentation in the negative control at 0 h (lane 1). Some background apoptosis is visible in the negative control at 6 h of incubation (lane 2); intense DNA fragmentation is apparent in IBDV- (lane 3) and hydrocortisone-treated cells (lane 4). DNA size markers are shown in lane M.
Tris-HCl pH 7.4 and 1 mM-EDTA. Aliquots of 15 μl containing approximately 1500 ng of DNA were then electrophoresed in 1.5% agarose gels for 1.5 h at 60 V. Gels were stained with ethidium bromide and photographed under u.v. transillumination. Intense internucleosomal fragmentation of DNA, a pattern highly specific to apoptosis, was observed in PBLs treated with IBDV or with hydrocortisone (Fig. 2). DNA fragments corresponding to one to five nucleosomes were identified as bands by gel electrophoresis and longer fragments were also detected as a smear in the gel near the origin of migration. On the other hand, the gel of the negative control cells at both 0 h and 6 h of incubation showed a more intense smear near the origin of migration. The negative control at 6 h of incubation showed some weaker bands (background apoptosis), indicating that most of the DNA was still unfragmented as compared with the IBDV- and hydrocortisone-treated cells.

To substantiate further that IBDV-infected cells had undergone apoptosis, negative control and IBDV-infected PBLs were harvested and processed for electron microscopy. Ultrathin sections were examined in a Zeiss EM 10 transmission electron microscope (Carl Zeiss). Normal negative control cells are shown in Fig. 3(a). IBDV-infected PBLs displayed extensive peripheral chromatin condensation, zeiosis or blebbing of the plasma membrane (Fig. 3b) and the formation of apoptotic bodies (containing repackaged portions of the fragmented nucleus and an array of intact organelles such as mitochondria and lysosomes; Fig. 3c). However, there was no leakage of any of the cellular components into the extracellular space.

This study provides evidence that in vitro infection of avian PBLs with IBDV causes cellular DNA fragmentation, increases the cellular apoptotic and necrotic indices and induces the condensation of nuclear chromatin and the formation of apoptotic bodies. These features support the hypothesis that IBDV causes PBLs to undergo apoptosis.

Although immunosuppression is a well documented consequence of IBDV infection, the mechanism is still not well characterized. Since the virus exerts a cytopathic effect on immature B cells in the medullary area of the bursal follicles (Müller, 1986; Burkhardt & Müller, 1987), and induces direct cytolysis not only in bursal cells but also in thymic cells and in PBLs (Lam, 1988), it seems reasonable to suggest that there is direct virus-induced lympholysis which should result in an extensive

Fig. 3. Electron micrographs of peripheral blood lymphocytes. (a) PBL from the negative control. (b) Apoptotic PBL. (c) Apoptotic bodies (arrows) around an apoptotic cell from the IBDV-treated group. Bar markers represent 1 μm.
inflammation of the BF, severe lymphoid depletion and suppression of the humoral response. However, there are reports of IBDV causing immunosuppression in chickens and yet causing little or no inflammatory response in the BF (Rosenberger et al., 1985; Sharma et al., 1989; Snyder, 1990; Lukert & Saif, 1991). Therefore, virus-induced necrosis alone may not be responsible for the immunosuppression. Atrophy without inflammation is one of the characteristics of apoptosis. We have shown that exposure of chicken lymphocytes to IBDV can induce apoptosis with all its associated characteristics: DNA fragmentation, nuclear and cellular segmentation, and apoptotic body formation. These findings suggest that IBDV-induced immunosuppression may be caused, at least in part, by the activation of programmed cell death in lymphocytes.

References


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