Bovine viral diarrhoea virus and bovine herpesvirus-1 prime uninfected macrophages for lipopolysaccharide-triggered apoptosis by interferon-dependent and -independent pathways

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The flavivirus bovine viral diarrhoea (BVD) virus exists in two biotypes, cytopathic (cp) and non-cytopathic (ncp), defined by their effect on cultured cells. Cp BVD virus-infected cells undergo apoptosis and may promote apoptosis in uninfected cells by an indirect mechanism. Macrophages (Mφ) infected with cp, but not ncp, BVD virus release a factor(s) in the supernatant capable of priming uninfected Mφ for activation-induced apoptosis in response to lipopolysaccharide. A possible role of interferon (IFN) type I was suggested previously by the observation that this cytokine primed for activation-induced apoptosis and was present in supernatants of Mφ infected with cp, but not ncp, BVD virus. Here, supernatants of both Mφ infected with a wider range of cp BVD virus and Mφ infected with bovine herpesvirus-1 are shown to contain such priming activity. Two lines of evidence indicate that factors in addition to IFN type I prime uninfected Mφ for apoptosis. First, supernatants of Mφ infected with cp BVD virus contained much less IFN than is required for priming for apoptosis. Second, whereas antiviral activity was neutralized by a vaccinia virus-encoded IFN type I receptor, B18R, the capacity of the supernatant to prime for apoptosis was unaffected by this treatment. The apparent molecular mass of the factor(s) priming for apoptosis was between 30 and 100 kDa. Priming of uninfected cells for activation-induced apoptosis may add a new facet to virus pathogenesis and may contribute to the formation of lesions not related directly to virus replication.

Introduction

Viruses have evolved strategies to induce or prevent apoptosis in their host cells. Generally, virus-induced apoptosis is believed to enhance the release of virus progeny from infected cells. It may, however, also represent a host defence mechanism, as apoptotic cells cease to produce virus progeny. Virus inhibition of apoptosis may therefore contribute to prolonged infection (for reviews see Knipe, 1996; Evan & Littlewood, 1998; O’Brien, 1998; Tschopp et al., 1998).

Bovine viral diarrhoea (BVD) virus is a pestivirus of the family Flaviviridae (Baker, 1987; Nettleton & Entrican, 1995). This virus is of particular interest in studies of virus-induced apoptosis because of the existence of closely related ‘pairs’ of cytopathic (cp) and non-cytopathic (ncp) biotypes (Weiss et al., 1994; Paton, 1995). The two biotypes of such a pair differ only in one non-structural protein, NS23, which is cleaved into NS2 and NS3 in cp, but not ncp, biotypes (Brownlie, 1990; Meyers & Thiel, 1996). Moreover, both biotypes of BVD virus are associated with mucosal disease, a lethal form of infection with BVD virus. It is observed in animals infected persistently with ncp BVD virus from the early period of their intrauterine development. The cp biotype of BVD virus detected in animals with mucosal disease may be the result of a mutation of the ncp biotype (hence the term ‘virus pair’), or may be due to superinfection. The cp biotype is believed to kill these immunotolerant animals because their immune systems fail to control virus multiplication (Brownlie, 1990; Meyers & Thiel, 1996). Cells infected with cp BVD virus have been shown to

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undergo apoptosis (Zhang et al., 1996), which is associated with cleavage of poly(ADP-ribose) polymerase (Hoff & Donis, 1997; Schweizer & Peterhans, 1999) and can be prevented by selected antioxidants (Schweizer & Peterhans, 1999). In addition to inducing apoptosis in its host cells, BVD virus may also influence the viability of uninfected cells, via factors released from infected cells. An example of such factors are interferons (IFNs), which have been shown to have anti-apoptotic properties (Rodriguez-Villanueva & McDonnell, 1995; Yanase et al., 1998). Specifically, IFNs have been shown to prime for apoptosis via increased synthesis of 2',5'-oligoadenylate (2-5A) and its activation of RNase L (Der et al., 1997; Diaz-Guerra et al., 1997; Castelli et al., 1998) or via inhibition of protein synthesis by PKR (Jagus et al., 1999). Apoptosis of uninfected NIH3T3 cells was observed when RNase L was overexpressed by means of an inducible promoter or activated by 2-5A or double-stranded RNA (Castelli et al., 1998). In contrast, inhibition of the 2',5'-oligoadenylate synthetase/RNase L system prevented apoptosis in response to double-stranded RNA (Rivas et al., 1998). Pre-treatment of mice with IFN-γ was shown to enhance lipopolysaccharide (LPS)-triggered apoptosis markedly in thymocytes, whereas the simultaneous injection of anti-IFN-γ antibody and LPS prevented apoptosis (Kato et al., 1997). In vitro, human immunodeficiency virus type 1 (HIV-1)-infected macrophages (Mφ) enhanced dramatically the proportion of cells undergoing apoptosis of uninfected but not infected T cells (Herbein et al., 1998). These authors postulate an indirect mechanism of apoptosis in uninfected T cells mediated by antigen-presenting cells. Furthermore, in porcine reproductive and respiratory syndrome virus infection, it is mainly uninfected bystander cells that undergo apoptosis, rather than infected cells. In vivo studies showed the apoptotic cells to consist predominantly of mononuclear cells (Sirinarumitr et al., 1998).

Supernatants of Mφ infected with cp BVD virus that prime uninfected Mφ for apoptosis were found to contain IFN (Adler et al., 1997). Moreover, recombinant bovine (rbo) IFN-α1 primes for apoptosis (Adler et al., 1995). Therefore, we investigated whether it is the IFNs that are responsible for priming by supernatants of cp BVD virus-infected Mφ. We showed that priming was due to a factor(s) distinct from, or in addition to, IFNs. The presence of such a factor(s) in supernatants of Mφ infected with different strains of cp BVD virus and bovine herpesvirus-1 (BHV-1), a cp DNA virus, suggests that priming for activation-induced apoptosis may be of more general significance in virus–host interactions.

Methods

Reagents. Foetal calf serum (FCS) and cell culture media for bovine cells were obtained from Seromed (Biochrom) and culture media for insect cells were bought from Life Technologies. FCS was free of BVD virus and antibody to BVD virus, as tested by virus isolation and serum neutralization assay, respectively. β-Propiolactone for inactivation of virus infectivity was purchased from Sigma. RboIFN-α was generously provided by Novartis.

Cells. Mφ were obtained from the blood of Red Holstein cows as described previously (Jungi et al., 1997). Briefly, peripheral bovine mononuclear cells were isolated by using Ficoll–Hypaque centrifugation and Mφ were permitted to differentiate by culturing the cells for 7 days under non-adherent conditions in Teflon bags at a cell concentration of 4 × 10⁶ cells/ml in Iscove's modified Dulbecco's minimum essential medium (MEM) supplemented with HEPES (10 mM), nonessential amino acids for MEM (Life Technologies, 1%), vitamins for MEM (Life Technologies, 1%), streptomycin (100 µg/ml), penicillin (100 IU/ml), amphotericin B (2.5 µg/ml), l-glutamine (2 mM), 2-mercaptoethanol (50 µM) and 20% FCS. Madin Darby bovine kidney (MDBK) cells and S9 insect cells were obtained from the American type culture collection (Manassas, VA, USA). MDBK cells of passages 120 to 140 were used for IFN type I assay. BVD and BHV-1 viruses were grown and titrated on bovine embryonic tubinate cells. These cells were prepared from foetuses obtained from a local abattoir and were maintained in MEM supplemented with 2% FCS, l-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified 5% CO₂ atmosphere. All batches of cells used in our experiments were found to be free of BVD virus by immunofluorescence testing.

Viruses. BHV-1 (Colorado strain) was originally obtained from V. Bitsch (Aarhus, Denmark). The recombinant baculovirus vector expressing B18R and the vector control were kindly provided by G. L. Smith (Sir William Dunn School of Pathology, Oxford, UK) and have been described elsewhere (Alcami & Smith, 1992; Symons et al., 1995). The BVD virus strains used included NADL, TGAC (Ridpath et al., 1991) and SuwaCP (all cp) and TGAN (Ridpath et al., 1991) and SuwaNCP (both ncp). The TGAC and TGAN virus strains were kindly provided by V. Moenig (Hannover, Germany), whereas SuwaCP and SuwaNCP are a ‘virus pair’ isolated from peripheral blood of an animal suffering from mucosal disease. Separation and biological cloning of the two Suwa BVD virus biotypes was performed by the plaque formation method using a methyl cellulose overlay medium consisting of 1.5% methyl cellulose in MEM supplemented with penicillin (100 IU/ml) and 2% FCS (Nakamura et al., 1993). The genetic relationship of SuwaCP and SuwaNCP BVD viruses was determined by sequencing using standard techniques and an ABI PRISM 310 Genetic analyser (Perkin Elmer). The following primers were used for amplification of BVD virus cDNA: 5′-UTR, 5′ GAGGCTA-GCCATGCCCCCTAG 3′ (sense) and 5′ TCAACTCTGATGCCCCAT-GTAC 3′ (antisense); E2, 5′ GCAGGCTATGCTAGTACTA 3′ (sense) and 5′ GCCCTCCATCTGATGAT 3′ (antisense); and NS23, 5′ GAACTCGGCCCATGGCAAA 3′ (sense) and 5′ ACTGGGCT-CTGGGTGTTGTG 3′ (antisense). Analysis of the sequences revealed that, with the exception of four point mutations in the NS23 region, the nucleotide sequences of the two virus genomes were identical. One of these mutations was silent, whereas the other three led to changes in the amino acid sequence. Northern blotting of genomic viral RNA performed by hybridization with virus strain-specific DIG-labelled RNA probes revealed that the genomes of SuwaCP and SuwaNCP BVD viruses were both approximately 12.5 kb in size (results not shown).

Induction of apoptosis. Mφ seeded in 25 cm² culture flasks at a density of 5 × 10⁶ cells/ml were infected with the appropriate strain of BVD virus or BHV-1 in 1 ml culture medium without FCS at an m.o.i. of 1 for 1 h at 37 °C. After adsorption of the virus, the inoculum was removed by washing the cells in culture medium without FCS prior to the addition of complete medium with 2% FCS. Supernatants were harvested after 48 h incubation at 37 °C and virus was inactivated by incubation...
with β-propiolactone at a 4000-fold dilution for 18 h at 4 °C followed by 2 h at 37 °C (Barrett et al., 1984; Perler et al., 1999). Virus inactivation was verified by titration on bovine turbinate cells. Mφ seeded in 24 well plates at a density of 5 x 10⁵ cells per well were incubated with the inactivated supernatant (400 μl per well) and fresh culture medium with 2% FCS (400 μl per well) for 48 h at 37 °C. The inoculum was replaced by fresh culture medium containing 1 μg/ml LPS. Apoptosis was measured after 48 h incubation.

**IFN assay.** Procedures for measuring biological activity of type I IFN in supernatants of virus-infected bovine monocyte-derived Mφ were described previously. The assay used is based on the reduction of Sendai virus growth by IFN as determined by immunocytochemistry (Perler et al., 1999).

**Apoptosis assay.** The fragmentation of cellular DNA was analysed quantitatively by FACS analysis according to Cossarizza et al. (1995). Detached cells were collected by centrifugation and the pellet was lysed with 500 μl lysis buffer (0.1 M sodium citrate, pH 6.5, 1% Triton X-100 and 25 μg/ml propidium iodide), resulting in the release of nuclei by nonadherent cells. The lysate was then added to the plate to release the nuclei of the remaining adherent cells. Nuclei were analysed with a FACScan flow cytometer (Becton Dickinson) after 30 min incubation at 4 °C in the dark. A minimum of 10⁴ nuclei per sample was analysed.

**Virus titration.** Viruses were passaged and titrated in bovine turbinate cells as described previously (Adler et al., 1994) and the titres of the virus stocks and supernatants were calculated according to Reed & Muench (1938).

**Results**

**Induction of IFN**

The TGAC strain of BVD virus was shown to induce IFN type I in bone marrow-derived Mφ (Adler et al., 1997). In the present study, this effect was also observed in bovine monocyte-derived Mφ infected with the cp TGAC, SuwaCP and NADL strains of BVD virus. Taking rboIFN-α1 as the standard, the mean concentration of IFN was found to range between 130 and 400 pg/ml, depending on the virus strain used to infect Mφ (Fig. 1). No antiviral activity was detected in supernatants of Mφ infected with ncp BVD virus (TGAN, SuwaNCP) or those of uninfected Mφ. However, the concentrations of IFN type I induced by cp BVD virus were rather low in comparison with those induced by infection with BHV-1, which were two to three orders of magnitude higher (Fig. 1).

**Activation-induced apoptosis**

Inactivated supernatants of cp BVD virus-infected Mφ were capable of priming uninfected Mφ for activation-induced apoptosis in response to LPS as quantified by the appearance of subgenomic DNA (Figs 2 and 3). The activity that primed for LPS-induced apoptosis was clearly restricted to supernatants of cp BVD virus-infected Mφ (NADL, SuwaCP, TGAC). Induction of apoptosis was not observed with supernatants of ncp BVD virus-infected Mφ (SuwaNCP, TGAN). The percentage of nuclei with subgenomic DNA induced by undiluted supernatants ranged from 13 to 55% depending on the virus strain used (Fig. 3). In comparison, a dose of 2000 pg/ml recombinant bovine IFN-α1 primed for 15% (Fig. 4b) and a dose of 20000 pg/ml primed for only 26% (result not shown). Priming for activation-induced apoptosis was also observed with supernatants of Mφ infected with BHV-1. These supernatants were markedly more potent in priming for apoptosis than those of Mφ infected with the NADL strain, followed by the TGAC and SuwaCP cp BVD virus strains (Fig. 3), which parallels the capacity of these strains to induce IFN type I in Mφ (Fig. 1). However, the extent of priming for activation-induced apoptosis could not be explained by their antiviral, i.e. IFN-like, activity.

**Dissociation of priming for apoptosis from IFN type I activity**

IFN type I produced by monocyte-derived Mφ in response to virus infection consists of a mixture of the IFN type I subfamilies (Ommann et al., 1987; Sager et al., 1998) and, therefore, its effect on target cells may differ from that of rboIFN-α1 at equivalent concentrations. To obtain more direct information on the role of IFN present in supernatants of infected Mφ in priming for activation-induced apoptosis, we investigated the effect of a soluble receptor of IFN type I encoded by the B18R gene of vaccinia virus (Alcami & Smith, 1992). B18R dramatically decreased the antiviral activity of IFN type I, regardless of whether it was due to rboIFN or natural bovine IFN type I produced by Mφ in response to infection with cp BVD virus or BHV-1 (Fig. 4a). Accordingly, B18R inhibited priming for LPS-triggered apoptosis by rboIFN-α1. In contrast, the soluble IFN receptor B18R had no effect on priming for activation-induced apoptosis by supernatants of Mφ infected with cp BVD viruses (Fig. 4b). The soluble IFN receptor partially reduced priming by supernatants
Fig. 2. Flow-cytometric analysis of activation-induced apoptosis. Bovine monocyte-derived Mφ were incubated with supernatants of TGAC cp BVD virus-, TGAN ncp BVD virus- or mock-infected Mφ. After a second incubation with LPS, DNA fragmentation was quantified by FACS analysis as described in Methods. The FACS histograms show a representative experiment, with propidium iodide fluorescence detected in FL1 (x-axis) and the number of nuclei given on the y-axis.

Fig. 3. Activation-induced apoptosis. Bovine monocyte-derived Mφ were incubated with inactivated supernatants of cp BVD virus- (NADL, TGAC, SuwaCP), ncp BVD virus- (TGAN, SuwaNCP), BHV-1- or mock-infected Mφ were incubated either with a soluble receptor for IFN type I (B18R, open bars) or with similarly treated product from the empty vector (shaded bars). Supernatants of BHV-1-infected Mφ contained high concentrations of IFN type I and were, therefore, diluted 1:1000. (a) The remaining biological activity of IFN type I was measured as described in Fig. 1. IFN type I is expressed in concentrations of the standard, rboIFN-αI.1 (mean ± SD, n = 4). (b) The priming activity for activation-induced apoptosis in response to LPS was analysed by flow cytometry as described in Fig. 2 (mean ± SD, n = 4).

Characteristics of the priming activity

A set of experiments was performed to characterize further the factor(s) contained in supernatants of cp BVD virus-infected Mφ responsible for priming for activation-induced apoptosis. The molecular mass was estimated by ultrafiltration of supernatants. Whereas filtration with membranes of an exclusion size of 100 kDa had no effect on the priming activity, a filter with a nominal exclusion size of 50 kDa eliminated the activity (not shown). Since many proteins with molecular masses of about 30 kDa are retained by the 50 kDa cut-off filters (Amicon), the molecular mass is between approximately 30 and 100 kDa. The priming activity of the supernatants was stable when exposed to pH 2 at 4 °C for 24 h, resisted heating at 56 °C for 30 min but not boiling for 15 min (Adler et al., 1997). Significant differences in the virus load between the two BVD virus biotypes may influence the release of the apoptosis-
Virus-induced factors prime for apoptosis

Discussion

The experiments reported here demonstrate that (i) in monocyte-derived Mφ, a range of cp, but not ncp, strains of BVD virus induce the production of a factor(s) capable of priming uninfected Mφ for activation-induced apoptosis; (ii) BHV-1 also induces such priming activity; and (iii) priming by supernatants of infected Mφ is due entirely (BVD virus) or largely (BHV-1) to factors distinct from IFN type I. The conclusion that IFN type I was of no, or only minor, importance for priming was reached, on the one hand, on the basis of a comparison between dose–response curves of antiviral and apoptosis-inducing activities and, on the other hand, on the failure of a soluble receptor of IFN type I to block apoptosis, despite its neutralization of antiviral activity.

IFN type I was initially considered to be responsible for priming for apoptosis because, in infected Mφ, only BHV-1 and cp, but not ncp, BVD viruses induced IFN type I, which, in its recombinant form, was shown to prime uninfected Mφ for activation-induced apoptosis (Adler et al., 1995, 1997). However, the mechanism of induction of IFN type I and of the priming factor(s) may be similar, which may explain the correlation between the ability of the different virus strains used to induce the formation of IFN type I and of the factor(s) that primes for LPS-induced apoptosis (Figs 1 and 3).

Only a few viruses, HIV (Finkel et al., 1995; Cottrez et al., 1997; Chen et al., 1998; Herbein et al., 1998), murine cytomegalovirus (Koga et al., 1994) and feline immunodeficiency virus (FIV) (Mizuno et al., 1997), have been reported to date to prime uninfected cells for activation-induced apoptosis. The priming for activation-induced apoptosis seems to be due to different mechanisms. HIV has been shown to prime T cell apoptosis by a factor(s) released from monocytes (Chen et al., 1998), but direct contact between infected and uninfected cells may also be involved (Cottrez et al., 1997). The observations made in FIV infection point to soluble mediators released from infected cells. The factor involved has not been characterized in any of these studies. Several studies have attributed a role of soluble factors in priming for apoptosis to certain cytokines, among them IL-4 (Mangan et al., 1992) and IFN-γ (Munn et al., 1995; Bingisser et al., 1996). This raised the question as to whether a previously described cytokine mediates priming. IFN-γ had to be considered, since in some of the blood-derived cultures both IFN-γ mRNA and bioactivity were detected. However, this signal was tentatively attributed to lymphocytes rather than to Mφ, since it was not detected in bone marrow-derived cultures of Mφ infected in a similar manner (M. Schweizer, unpublished observation). This suggests that the Mφ-derived priming factor is distinct from IFN-γ. Moreover, virus infection with both cp and ncp BVD viruses failed to induce detectable amounts of TNF-α, as determined by bioassay (Adler et al., 1998). This argues against an important role of this cytokine in priming for activation-induced apoptosis.

It remains to be shown whether the Mφ is the only cell type that produces a factor(s) capable of priming for apoptosis, and whether it is the only cell capable of being primed for activation-induced apoptosis. However, bovine turbinate cells produced neither IFN type I nor the priming factor in response to BVD virus or BHV-1 infection, nor could they be primed for LPS-induced apoptosis (results not shown). Recently, Lambot and co-workers showed that only the cp biotype of BVD virus induced apoptosis in bovine peripheral mononuclear cells. Directly relevant to this study is their observation that apoptosis in CD4+ and CD8+ T cells was enhanced significantly by the presence of monocytes (Lambot et al., 1998). Together with the observations reported for BHV-1 (Hanon et al., 1996; this work), HIV (Finkel et al., 1995), FIV (Mizuno et al., 1997) and murine cytomegalovirus (Koga et al., 1994), this suggests that, during virus infection, mononuclear cells may play a role in regulating apoptosis in an array of other cell types. The concept that apoptosis of uninfected cells may require at least two signals, i.e. one for triggering and one for triggering apoptosis, opens up new perspectives regarding the study of the biochemistry involved.

It will be of particular interest to investigate whether apoptosis controlled by mononuclear phagocytes is related to lymphopoiesis, a hallmark of most systemic virus infections. In addition, the increased susceptibility of cattle infected by BHV-1 to secondary bacterial infections, e.g. Pasteurella spp. (Bielefeldt Ohmann & Babiuk, 1985), may be related to activation-induced cell death. Moreover, ‘priming’ may not be restricted to mediating sensitivity to activation-induced apoptosis, but may include other functional changes in uninfected cells. Priming by endogenously produced IFN type I and by exogenously added IFN-αβ down-regulated IFN-γ- and TNF-α-induced nitric oxide production in mouse Mφ infected by tick-borne encephalitis virus and in uninfected Mφ, respectively (Kreil & Eibl, 1995). The immunosuppression observed during measles is another example of such functional changes. It seems remarkable that an as yet unidentified factor(s) released from one B lymphocyte sufficed to impair antigen presentation by some 120 B lymphocytes (Fujinami et al., 1998; Sun et al., 1998). These findings suggest that ‘priming’ may play an important role in the pathogenesis of virus infections.

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