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# Macrophages Infected with Cytopathic Bovine Viral Diarrhea Virus Release a Factor(s) Capable of Priming Uninfected Macrophages for Activation-Induced Apoptosis

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**Bovine bone marrow-derived macrophages infected with the cytopathic biotype of bovine viral diarrhea virus released an antiviral activity into the supernatant which was tentatively characterized as type I interferon because of its physicochemical properties. Such supernatants primed both infected and uninfected macrophages for decreased nitric oxide production and apoptosis in response to lipopolysaccharide. This finding strongly suggests a role of this pathway in the pathogenesis of mucosal disease, a lethal form of infection with cytopathic bovine viral diarrhea virus in which the principal lesions are located in the oral cavity and the gastrointestinal tract, which are known to contain a high concentration of endotoxin.**

Bovine viral diarrhea virus (BVDV) belongs to the *Pestivirus* genus in the *Flaviviridae* family (27). BVDV is one of the most important pathogens of cattle, causing a variety of clinical symptoms, e.g., pyrexia, anorexia, diarrhea, and respiratory distress (20). In vivo, BVDV infects an array of different cell types, including cells of the immune system (24), which may lead to the immunosuppression commonly associated with BVDV infection (20). BVDV isolates can be divided into two biotypes, cytopathic (cp) and noncytopathic (ncp), according to their cytopathic effects in cell cultures. The two biotypes also play different roles in vivo. Infection of pregnant cows with the ncp biotype but not with the cp biotype can result in the birth of persistently infected calves which are immunotolerant to the infecting BVDV strain (6). Persistently infected animals can be superinfected with an antigenically related cp strain (6), or a cp biotype can arise in persistently infected animals due to genomic rearrangements (8, 17, 18, 21). Both lead to the most severe manifestation of BVDV infection, the so-called mucosal disease (MD), which is fatal to almost 100% of infected animals (5).

Typical of MD are erosive lesions in the oral cavity and the gastrointestinal tract, especially in Peyer's patches (28). Although MD is strictly associated with the cp biotype of BVDV, it has not been clearly demonstrated whether the cp biotype is cytopathic in vivo or whether these lesions are mediated by an indirect mechanism. We have recently shown that macrophages, one type of host cell of BVDV, are differentially affected in vitro by infection with cp or ncp biotypes of BVDV (2). Biotype-specific changes of cellular functions after infection may have an influence on biotype-specific differences in pathogenesis, especially in cells which are important mediators of antiviral host defense.

Here we show that in vitro infection of bovine bone marrow-derived macrophages (BBMM) with cp, but not with ncp, BVDV primes the cells for activation-induced apoptosis in the presence of lipopolysaccharide (LPS). We provide evidence that this effect might be mediated by an antiviral activity induced in the macrophages exclusively by infection with cp BVDV.

BBMM were obtained by culturing bone marrow cells collected from tibiae of fetal calves under nonadherent (Teflon bag) conditions as described previously (3). For virus infection and treatment with cell culture supernatants, cells cultured in suspension were harvested and subcultured in 25-$cm^2$ flasks ($2.5 \times 10^6$ cells), 96-well plates ($1 \times 10^5$ cells/well), or 24-well plates ($5 \times 10^5$ cells/well). Macrophages were infected with viral strains TGAN and TGAC, a pair of BVDVs isolated from an animal suffering from MD (22). TGAN is an ncp and TGAC is a cp biotype. For comparative studies a type I interferon (IFN) inducer, bovine herpesvirus 1 (BHV1; strain Colorado) was used. Adherent BBMM were infected at a multiplicity of infection of 1. The cells were incubated for 60 min at 37°C to allow adsorption of the virus. The virus inoculum was removed prior to the addition of complete medium. The fetal calf serum and one sample from each bone marrow isolation were screened for BVDV by cell culture and subsequent immunoperoxidase staining for viral antigen (2), and only BVDV-negative fetal calf serum and bone marrow cell preparations were used for the experiments.

We recently showed that infection of BBMM with ncp BVDV primes the macrophages for enhanced generation of nitric oxide (NO) upon triggering with LPS, whereas infection with cp BVDV downregulates LPS-induced NO production (2). This difference was not due to a different susceptibility of macrophage cultures to infection with cp or ncp BVDV (2). Since these effects on NO production were seen with all BVDV strains used, we chose one virus pair (TGAN and TGAC) for this study. To elucidate the possible mechanism(s) responsible for these virus-induced changes in a pathway implicated in antimicrobial defense by macrophages (16, 19, 25) we looked for the involvement of a soluble factor(s). Macrophages were treated with virus-free supernatants of BVDV-infected macrophage cultures. Supernatants from virus-infected BBMM cultures were harvested 48 h after infection, and either the supernatant was filtered through molecular filters (Microcon 100; Amicon, Beverly, Mass.) to remove virus particles or the virus was inactivated by UV light (UV lamp, 15 W; Philips, Eindhoven, The Netherlands) at a distance of 15 cm for 10 min or by boiling the supernatants for 30 min. Complete removal or

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TABLE 1. Supernatants of cp BVDV-infected macrophage cultures contain NO-downregulating activity[a]

| Infection | $NO_2^-$ concn (μM) in supernatant[b] | | | |
|---|---|---|---|---|
| | Containing live virus | UV inactivated | Filtered | Boiled |
| Mock | 70.3 ± 5.49 | 68.8 ± 1.70 | 60.6 ± 3.05 | 43.6 ± 4.80 |
| ncp BVDV | 85.4 ± 3.72[c] | 66.1 ± 4.91 | 59.7 ± 4.00 | 50.2 ± 2.86 |
| cp BVDV | 13.6 ± 1.54[d] | 25.4 ± 2.95[d] | 25.2 ± 2.30[d] | 45.9 ± 2.81 |

[a] BBMM were cultured in microtiter plates, treated for 48 h with the indicated supernatants, and subsequently stimulated for 24 h with LPS (1 μg/ml). Then supernatants were collected for nitrite determination by using the Griess reagent.

[b] Supernatants from BVDV-infected BBMM were either left untreated or treated as described in the text. Values are means ± standard deviations of triplicate wells from one of five representative experiments.

[c] Significantly different from value for mock-infected cultures ($P < 0.02$; Student's $t$ test).

[d] Significantly different from value for mock-infected and ncp BVDV-infected cultures ($P < 0.002$; Student's $t$ test).

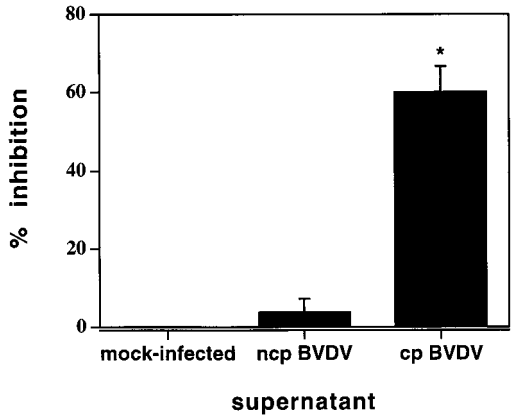


FIG. 1. Detection of antiviral activity in supernatants of BVDV-infected BBMM. BBMM were either mock infected or infected with BVDV. Supernatants were harvested 48 h after infection, UV inactivated, and tested for antiviral activity. The asterisk indicates that only supernatants of cp BVDV-infected macrophages contained a significant level of antiviral activity ($P < 0.001$, Student's $t$ test). The results shown are means of four independent experiments ± standard errors of the means.

inactivation of infectious viral particles was confirmed by virus isolation in cell culture. The production of NO by BBMM after LPS activation was determined by measuring nitrite in cell-free culture supernatants by using the Griess reagent (4). Priming for enhanced generation of NO by ncp BVDV was abolished by removing infectious virus, suggesting a direct virus effect (Table 1). In contrast, the downregulating activity of cp BVDV infections was preserved in virus-free or UV-inactivated supernatants and was destroyed only after the supernatants had been boiled (Table 1). This suggested that the observed NO downregulation was due to a soluble factor and not, as might be expected, to a cytopathic effect of the cp biotype. Indeed, different cp BVDV virus strains tested did not show a pronounced cytopathic effect in bovine macrophages (2) (data not shown).

One factor capable of downregulating NO production in bovine macrophages after LPS induction is type I IFN (1). We therefore screened the cell culture supernatants for antiviral activity. Antiviral activity was determined by the ability of test samples to inhibit the replication of Sendai virus in Madin-Darby bovine kidney cells as described previously (1). The presence of antiviral activity was expressed as percent inhibition of viral growth compared to the growth of the virus control (Fig. 1). Only supernatants from cp BVDV-infected macrophages but not from ncp BVDV-infected cells contained antiviral activity (Fig. 1), which could be tentatively characterized as type I IFN by its stability at a pH of 2 (24 h, 4°C) and at 56°C (30 min) and by its being nondialyzable (data not shown). Recombinant bovine alpha interferon (IFN-α) and supernatants from macrophages infected with BHV 1, known to be a strong type I IFN inducer (9), showed identical physicochemical properties (data not shown).

We recently showed that pretreatment of bovine macrophages with recombinant IFN-α primed the cells for LPS-induced apoptosis and that cell death was accompanied by downregulation of LPS-induced NO production (1). Similarly, pretreatment with supernatants of cp BVDV-infected macrophage cultures led to a concentration-dependent increase in cell death after LPS stimulation, whereas supernatants from mock-infected or ncp BVDV-infected macrophages did not affect the viability of LPS-activated macrophages (Fig. 2A). Cell viability was measured by staining adherent cells for 10 min with 0.75% crystal violet in a solution of 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde. Stained cells were lysed in isopropanol, and the crystal violet intensity was measured at a λ of 590 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Cell death was identified as being apoptotic (Fig. 2B) by using a cell death detection ELISA (Boehringer Mannheim, Mannheim, Germany), which measures fragmented DNA in the cytosolic fraction of the cells. The corresponding unfragmented nuclear DNA was measured chemically in a reaction with diphenylamine (7). DNA fragmentation was expressed in arbitrary units as the ratio of fragmented to unfragmented DNA. Cell death measured with crystal violet correlated with apoptosis measured by using the cell death detection ELISA (correlation coefficient, >0.9). NO production decreased, and cell death of macrophages treated with supernatants of cp BVDV-infected cells increased in parallel with the antiviral activity in the cell culture supernatants (Fig. 2C to E). Thus, macrophage function and viability are affected similarly by supernatants from cp BVDV-infected macrophages and by recombinant bovine IFN-α- or BHV1-induced type I IFN (Table 2), implying that cp BVDV-induced antiviral activity plays a role in priming macrophages for activation-induced cell death.

This is the first demonstration that BVDV induces an antiviral activity in macrophages and that this effect of viral infection is restricted to the cp biotype. Another important finding is the capacity of supernatants of cp BVDV-infected macrophages to prime uninfected macrophages for LPS-induced apoptosis. These findings could be relevant for the pathogenesis of infection with BVDV. Thus, rapid induction of IFN by the cp biotype could be one reason that this virus fails to establish a persistent infection in the fetus. Indeed, it has been demonstrated that infection of pregnant cows with cp BVDV during a period of gestation during which an ncp biotype can establish persistent infection leads to IFN induction both in the cow and in the fetus (23). For the ncp biotype an induction of IFN in vivo has not been demonstrated. In addition, we found that the antiviral activity produced by cp BVDV-infected macrophages could block infection of bovine turbinate cells in vitro with either biotype (data not shown). Furthermore, it has been shown that an infection of calves with the cp biotype of BVDV via the respiratory route led to a general impairment of alveolar macrophage functions, although only a small percentage of the macrophages became infected (26). This finding also

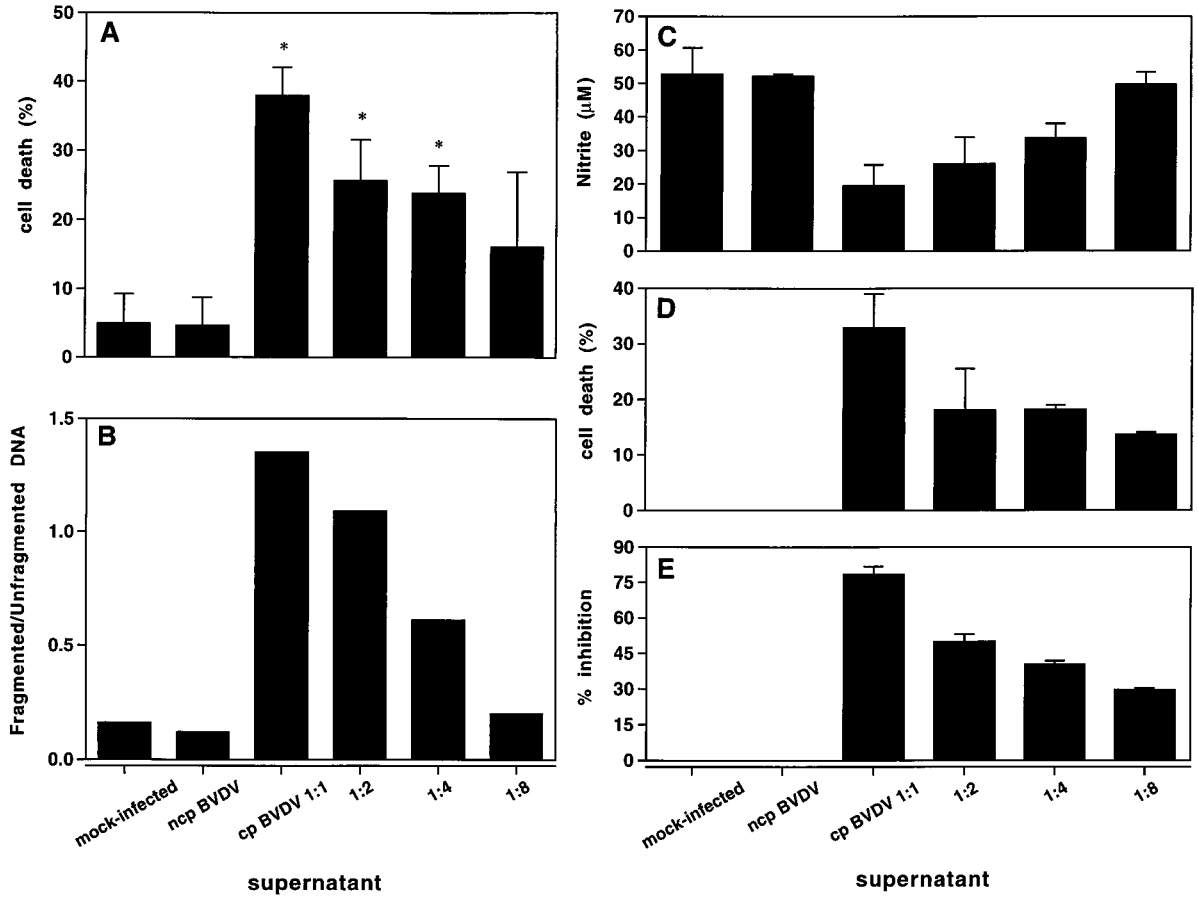


FIG. 2. Activation-induced cell death (A) and DNA fragmentation (B) in BBMM pretreated with supernatants from BVDV-infected macrophages. BBMM were pretreated for 48 h with UV-inactivated supernatants of mock-infected or BVDV-infected macrophages and subsequently stimulated for 24 h with LPS (1 μg/ml). (A) Cell death was measured by staining adherent cells with crystal violet. Results are expressed as percent cell death compared with that for macrophages pretreated with culture medium. Values marked by asterisks are significantly different from those for supernatants from mock-infected or ncp BVDV-infected BBMM ($P < 0.05$, Student's $t$ test). The results shown are means of four independent experiments ± standard errors of the means (B). Fragmented DNA was measured by using a commercial cell death detection ELISA. DNA fragmentation was expressed in arbitrary units as the ratio of fragmented to unfragmented DNA. One representative experiment of three is shown. (C, D, and E) Direct comparison of the capability of supernatants of BVDV-infected macrophage cultures to downregulate NO production and to induce cell death in LPS-stimulated macrophages and the antiviral activity in the same supernatants, respectively. Results of one representative experiment are shown (values are means ± standard deviations of triplicates). Supernatants from mock-infected or ncp BVDV-infected macrophage cultures neither caused detectable cell death (D) nor contained detectable antiviral activity (E).

suggests that a soluble factor affects the uninfected macrophages.

Whether the antiviral activity is the only factor responsible for priming uninfected cells for apoptosis and decreasing inducible NO synthase activity in response to LPS remains unclear. The titer of antiviral activity correlates with the extent of priming for apoptosis and the decrease in inducible NO synthase activity, and we recently demonstrated that this is also the case with recombinant bovine IFN-α-I-1 (1). While this would be consistent with an important role of IFN-α in mediating these effects, direct proof would depend on blocking experiments using neutralizing antibodies to natural bovine type I IFN, which are currently not available. There are indications that other viruses can influence macrophage functions via IFN-α/β. Tick-borne encephalitis virus, which is also a flavivirus, downregulates IFN-γ- and tumor necrosis factor al-

TABLE 2. Effects of recombinant bovine IFN-α and supernatants of BHV 1-infected macrophages on BBMM

| Supernatant[a] | Nitrite concn (μM)[b] | % Cell death[b] | Antiviral activity (% inhibition)[b] | Fragmented/unfragmented DNA ratio[c] |
|---|---|---|---|---|
| Control | 52.7 ± 7.9 | 0 | 0 | 0.2 |
| IFN-α | 5.4 ± 1.7 | 73.6 ± 2.9 | 90.2 ± 0.7 | 3.0 |
| BHV 1 | 5.9 ± 3.4 | 88.3 ± 1.3 | 84.3 ± 2.8 | 2.9 |

[a] BBMM were cultured in 24- or 96-well plates, treated for 48 h with recombinant bovine IFN-α (10 ng/ml) or UV-inactivated supernatants of macrophages infected with BHV 1 (a strong inducer of type I IFN), and stimulated for 24 h with LPS (1 μg/ml). Control, supernatant of mock-infected BBMM.

[b] Values are means ± standard deviations for duplicate or triplicate wells.

[c] Values are means from a single experiment.

pha-stimulated production of NO in mouse macrophages via IFN-α/β (13). Herpes simplex virus I induces in murine macrophages a factor(s) which suppresses their tumoricidal activity after IFN-γ stimulation (12), a block which can also be achieved by recombinant IFN-α in this system. A factor induced by BHV 1 in mononuclear cells leads to apoptotic death of these cells after mitogen stimulation (11). In the cases of tick-borne encephalitis and herpes simplex virus it remains to be shown whether changes in macrophage function are also due to activation-induced cell death.

Priming for apoptosis in response to LPS could also explain a poorly understood aspect of the pathology of MD. MD is correlated with the appearance of a cp BVDV in animals that are persistently infected with an ncp BVDV as a result of infection in utero. The cp biotype may arise by mutation from the ncp biotype, or it may be due to superinfection with a cp BVDV that is antigenically very similar to the persisting ncp BVDV. The lesions of MD are explained as damage exerted by the cp biotype (6, 10, 14), which is not restrained because it is tolerated by the immune system. In animals suffering from MD, cp BVDV can be readily isolated from many different organs in which no, or only slight, lesions are found (10, 15, 24a). It is surprising, therefore, that the typical lesions of MD are restricted to the oral cavity and the gastrointestinal tract, suggesting that the current explanation that the lesions are directly caused by cp BVDV is insufficient. Priming for LPS-induced apoptosis would offer a pathogenetic mechanism which could explain the location of the lesions in the oral cavity and the gastrointestinal tract. Thus, while this explanation does not rule out that part of the damage may be due to direct cell killing by the virus, it would provide a reason for the striking location of the lesions in a region with high endotoxin concentration close to the site of replication of cp BVDV.

In summary, the observations reported in this paper suggest that cytokines, in particular type I IFNs, may in fact contribute to the pathogenesis of mucosal disease. It seems remarkable that a mechanism which is generally viewed as beneficial for the host may cause lesions in immunotolerant animals as a result of the interaction with bacteria or bacterial constituents.

B.A. and H.A. equally contributed to this study.

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