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Influence of Interferons α,1 and γ and of Tumour Necrosis Factor on Persistent Infection with Bovine Viral Diarrhoea Virus in vitro

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SUMMARY

Non-cytopathic strains of bovine viral diarrhoea virus (BVDV) readily establish persistent infections in cells of bovine origin. The involvement of endogenous interferon (IFN) on the maintenance of the infection level, as well as the effect of exogenous IFN and tumour necrosis factor alpha (TNF-α), was studied. Although exogenous IFN suppressed the spread and replication of virus, it did not cure the infection, even when continuously present over many cell passages. TNF-α alone had no antiviral effect in this system. However, both TNF-α and IFN enhanced the cytopathic effect of cytopathic BVDV, and induced a BVDV-like cytopathic effect in cells infected with non-cytopathic BVDV. These data are discussed with regard to possible mechanisms of pathogenesis during fatal BVDV disease in cattle.

The mechanisms involved in virus persistence are multifarious and, in most cases, remain ill understood (Mims, 1986). Among the many factors claimed to be involved in the establishment and maintenance of persistent virus infections are the interferons (IFNs), either as mediators of persistence (Jacobson & McFarland, 1982) or because of lack of IFN production (Ikeda et al., 1986). One of the unresolved problems in animal virology is the pathogenesis of mucosal disease in cattle caused by bovine viral diarrhoea virus (BVDV). It is now established that the fatal disease occurs only in cattle which are persistently infected with and immunotolerant to a non-cytopathic (ncp) variant of BVDV. Precipitation of clinical symptoms may occur due to superinfection of such persistently infected animals with a cytopathic (cp) BVDV strain (Brownlie et al., 1987). However, there have also been observations to suggest that lesions and clinical disease are, at least in part, the outcome of immunopathological mechanisms (Bolin et al., 1985; H. Bielefeldt Ohmann, unpublished data). Since IFNs could potentially be involved in all these aspects (Preble & Friedman, 1983) as well as in the establishment of virus persistence (Mims, 1986) it was of interest to explore the influence of IFNs on BVDV infection. In view of a possible immunopathological involvement, the indications of macrophage activation during persistent BVDV infection (Bielefeldt Ohmann et al., 1987), as well as the recently documented antiviral effect of tumour necrosis factor alpha (TNF-α), produced by activated macrophages (Campos et al., 1987), studies were included to determine what effect TNF might have on BVDV infection. The present study took advantage of the persistent infection with ncp BVDV which can be readily established in vitro in cells of bovine origin.

Georgia bovine kidney (GBK) cells were used throughout this study as a source of BVDV persistently infected cells. Approximately 30 to 40% of the cells were positive for BVDV antigens, as determined by immunostaining, and continuously produced infectious ncp BVDV. Moreover, the cells could not be cured of infection by growth in a medium containing antiviral antibody. BVDV-free MDBK cells were used to study the effect of IFNs on an exogenous BVDV infection. For the latter purpose we used two biotypic variants, an ncp
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(10039/D7E10G10) and a cp (Ho4943/A5H7B10) strain obtained by three to five passages at limiting dilution from Danish field isolates of BVDV. The homogeneity of the clones was assessed by SDS–PAGE and Western blotting (Laemmli, 1970; Towbin et al., 1979; Bielefeldt Ohmann, 1988a). All cell cultivations were done in MEM supplemented with 10% horse serum for outgrowth and 2% horse serum during maintenance. Viral infection was determined by immunoperoxidase staining of cytopsins of trypsinized cells or on cell monolayers fixed in situ in tissue culture plates (Bielefeldt Ohmann et al., 1987). Virus titrations were done in a microtitre system as previously described (Bielefeldt Ohmann et al., 1987). Cells were trypsinized, pelleted and fixed for electron microscopy (Bielefeldt Ohmann et al., 1987). The cytokines used, bovine IFN-α1, IFN-γ and TNF-α (rBoIFN-α1, rBoIFN-γ, rBoTNF-α) were produced by recombinant DNA techniques in Escherichia coli and were obtained from Ciba-Geigy. Antiviral titres, as determined by inhibition of vesicular stomatitis virus replication in MDBK cells and adjusted to international standards (according to the producer, Genentech, South San Francisco, Ca., U.S.A.), were 2 × 10⁷ units (U)/mg protein for rBoIFN-α1 and 1 × 10⁷ U/mg protein for rBoIFN-γ. No biological standard is yet available for bovine TNF-α and this was therefore used on a weight basis; it does, however, appear to have an antiviral activity equal to that of rBoIFN-γ (Campos et al., 1987). The antiviral activity of the three cytokines was also evaluated by their 2–5A oligoadenylate synthetase-inducing capacity (Campos et al., 1987).

To assess whether the restricted virus infection in persistently infected GBK cell cultures could be due to endogenous IFN induced by the virus, supernatants from cultures of various ages were tested for antiviral activity. No antiviral activity was found (data not shown). This result is corroborated by the absence of 2–5A synthetase activity in the cultures (Campos et al., 1987). In this context it should be mentioned that persistent BVDV infection of cell cultures does not inhibit the induction of this enzyme by exogenous IFNs (unpublished data). Cell growth in the continuous presence of antibodies to bovine IFN-α, IFN-β or IFN-γ also had no effect on the infection level (data not shown).

Persistently infected cultures were passaged every 3 to 4 days and the outgrowth medium was replaced after 24 h each time by medium containing IFN-α1, IFN-γ or TNF-α (various doses). With this regime, the virus yield (data not shown) and the frequency of cells which were virus antigen-positive (i.e. infected cells) slowly decreased in cultures exposed to the rBoIFN-α1 and rBoIFN-γ whereas there was no detectable effect on those cells exposed to rBoTNF-α (Fig. 1a).

The effect of the rBoIFNs was dose-dependent. However, the infection could not be eliminated, in the number of passages possible, before inhibition of growth by the IFNs, especially IFN-γ. Furthermore, if IFN therapy was withdrawn at the time when 1% or fewer cells contained viral antigen, virus replication and spread throughout the culture immediately resumed (Fig. 1b). In fact, the infection spread to nearly 100% of the cells within 2 to 3 days of termination of treatment, sometimes followed by a subsequent return to the steady-state level of 30 to 40% virus-positive cells. A similar pattern of suppression and resumption of infection was seen if cells were grown without trypsinization over an extended period of time of up to 32 days in the continuous presence of cytokines which were renewed every 3 days to maintain an activity level of 50 to 100 U/ml of medium (Fig. 2). Various approaches were taken to determine whether selection of either IFN-insensitive substrains of virus and/or IFN-insensitive cell clones were selected in the presence of cytokines. Neither event was observed (data not shown). Retreatment of cells that had been treated over a number of passages, followed by two or three passages without IFN-α1 or IFN-γ gave results similar to those for the initial treatment series (Fig. 1 and 2). Furthermore, no change in biotypic characteristics of the virus, i.e. from ncp to cp, was observed. This was further corroborated by the continuous absence of the 80K Mr protein, characteristic of cp strains (Donis & Dubovi, 1987), in virus-infected cells as revealed by SDS–PAGE and immunoblotting (not shown). However, when persistently infected cells were treated with IFN-α1 or IFN-γ (especially the latter), morphological changes were seen, which varied from giant cell formation to viral plaque-like lesions in the monolayers. The latter phenomenon was especially prominent in MDBK cells, in which a persistent infection was established with an exogenous ncp BVDV strain. Whether this phenomenon could have any...
Fig. 1. Effect of rBoIFN-α1 (▲), rBoIFN-γ (○) (100 U/ml) and rBoTNF-α (□) (10 μg/ml) on BVDV persistence in GBK cells. Untreated cells (●) were included as a control. (a) Cells were passaged every 3 to 4 days with renewed treatment 24 h later. Antigen-positive cells were detected by immunocytochemistry on cytospin preparations made at every passage. (b) Following four or five passages as described in (a), IFN treatment was withdrawn and cultivation was continued for a period during which time cultures were terminated and virus antigen expression was determined. The data depicted are a representative example from a series of three independent experiments.

Fig. 2. Suppression of replication and spread of BVDV in persistently infected GBK cells by continuous growth of the cells in vitro without trypsinization and in the continuous presence of rBoIFN-α1 (▲, ▲), rBoIFN-γ (○, ○) (100 U/ml) or rBoTNF-α (□, □) (10 μg/ml). On day 20 (arrow) treatment was withdrawn from cultures containing rBoIFN-γ (●) and rBoIFN-α1 (▲). The infection level was determined by immunocytochemistry on fixed monolayers or cytospin preparations of trypsinized cells. The data depicted are from a representative experiment in a series of three independently conducted experiments (10% variation between experiments). Untreated cells (●—●) were included as a control.

bearing to the pathological lesions seen in clinical disease of cattle remains to be studied. At the ultramicroscopic level, the typical features of BVDV-infected cells, such as membrane-bound vesicles containing virus particles, membranous structures and granular material (Bielefeldt Ohmann & Bloch, 1982; Bielefeldt Ohmann et al., 1987) were less prominent in cells treated with rBoIFN-α1 or rBoIFN-γ. Fewer mature virus particles and less proteinaceous material were
present, although there appeared to be an increase in aberrant particle forms and 'empty' membrane structures in IFN-treated cells.

It has been reported that following experimental infection of bovine foetuses in the first 3 months of gestation with a mixture of cp and ncp BVDV variants, only ncp virus can be re-isolated from the foetus or newborn calf (Done et al., 1980; Bielefeldt Ohmann et al., 1987). This seems to suggest that cp strains are more sensitive to the host defence mechanism(s) for which the IFNs are obvious candidates. To explore this possibility, MDBK cell monolayers were treated for 24 h with either rBoIFN-α1 or rBoIFN-γ (0.1 to 10³ U/ml) or TNF-α (0.01 to 100 μg/ml), followed by passage in microtitre culture plates, and simultaneously infected with either a cp or ncp BVDV (1 infection unit per 10 cells). Infection and re-establishment of the cultures were allowed for 12 to 24 h, after which time the medium was replaced by MEM containing 2% horse serum and cytokine at concentrations similar to those used during pre-treatment. Over the following 6 to 7 days, cultures were terminated daily for assessment of virus infection. To ensure the continued presence of the cytokines at the levels wanted, the media containing cytokines were renewed every second day. At the concentrations used, TNF had no demonstrable inhibitory effect on the replication of either cp or ncp BVDV. In contrast, the infection level was reduced by both rBoIFN-α1 and rBoIFN-γ in the concentration range of 10 to 10³ U/ml. At high IFN concentrations (> 500 U/ml) the cp and ncp strains were inhibited to a similar extent (from 80 to 90% infected cells to less than 5%). At lower doses (50 to 200 U/ml) replication of the cp strain was slightly more suppressed than that of the ncp strain. However, the infection was not cured with either virus biotype; moreover, the cytopathic effect of the cp strain appeared to be enhanced by all three cytokines. Notably, after day 3 a BVDV-like cytopathic effect also developed in cytokine-treated cultures infected with the ncp strain. This was not due to a change in biotype of the virus, as determined by SDS-PAGE and immunoblotting (see below). In contrast, rBoIFN-γ and rBoIFN-α1 caused very little, if any, cytotoxic/growth inhibitory effect in non-infected cultures even after 7 days of continuous growth in the presence of high concentrations of cytokines (5 × 10² to 10 × 10² U/ml). However, rBoTNF-α had a marked c.p.e. in non-infected cultures at the higher concentrations (1 to 100 μg/ml) used.

The data presented here clearly demonstrate that although IFNs have some inhibitory effect on BVDV production and spread, as also reported by others (Czarniecki et al., 1986), they cannot cure the cell cultures when used at non-cytotoxic concentrations; neither do the IFNs appear to play a role in maintenance of the persistent infection. However, it is premature to generalize from the present in vitro system to all tissue types in vivo (Bielefeldt Ohmann, 1988b). TNF-α appeared to have no antiviral effect on BVDV, neither did it consistently enhance the effect of rBoIFN-α1 or rBoIFN-γ on BVDV replication (data not shown). However, a notable observation was that both TNF-α and the IFNs could, in cultures infected with a ncp variant of BVDV, induce a c.p.e. which was morphologically very similar to that induced by cp BVDV. Since accumulation of activated macrophages is a prominent characteristic of lesions in clinical BVDV disease (Bielefeldt Ohmann, 1988b) and since ncp BVDV strains can induce TNF-α and IFN-α production in bovine macrophages (unpublished data), it is conceivable that these cytokines are involved in the development of pathological lesions during the course of mucosal disease. However, the temporal relationship between macrophage accumulation and development of lesions has to be elucidated before this contention can be tested. The present data do not support the notion that IFN(s) is a selection factor for the virus type reaching the bovine foetus. However, a more appropriate host cell system and other IFN species should be used to explore this aspect further.

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