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Parvoviruses are inefficient in inducing interferon-β, tumor necrosis factor-α, or interleukin-6 in mammalian cells

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Abstract. To investigate a possible role of cytokines in parvovirus-mediated suppression of tumorigenesis, we tested in cell culture whether parvoviruses are able to induce interferon (IFN)-β, tumor necrosis factor (TNF)-α or interleukin-6 (IL-6). Infection of rodent or human cells with the parvoviruses minute virus of mice (MVM), H-1 or adeno-associated virus (AAV) types 2 or 5 failed to induce expression of the luciferase or β-galactosidase reporter genes transfected into these cells as constructs containing an IFN-β promoter. Parvoviruses did weakly induce synthesis of TNF-α and of IL-6 in cell culture and could slightly enhance synthesis of these cytokines when induced by other agents. These in vitro data suggest that the rather unspecific tumor-suppressive properties of parvoviruses are unlikely to be attributable to stimulation of the synthesis of IFN, TNF or IL-6.

Introduction

Parvoviruses of vertebrates have a single-stranded DNA genome of about 5000 bp and can be divided into autonomously replicating viruses [such as H-1, B-19, minute virus of mice (MVM), Aleutian disease virus (ADV), mink enteritis virus (MEV)] and adeno-associated viruses (AAV), which depend for their replication on factors provided by helper viruses [adenoviruses, herpes viruses, or vacciniavirus; (Cukor et al. 1984; Siegl et al. 1985; Schlehofer et al. 1986)] or specific cellular conditions related to factors induced by genotoxic stress (Schlehofer et al. 1983; Heilbronn et al. 1985; Yakobson et al. 1987; Yalkinoglu et al. 1988; Berns and Bohensky, 1987).

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A unique property of the parvoviruses is their capacity to suppress tumors in vivo and to inhibit cellular transformation in vitro (Rommelaere 1990; Rommelaere and Tettersall 1990; Rommelaere and Cornelis 1991). The mechanism of the tumor-suppressive effects of parvovirus infections is not yet understood, but in the last few years several aspects of the interference of parvoviruses with cell transformation in vitro have been described: AAV infections inhibit mutagenesis (Schlehofer and Heilbronn 1990), inhibit carcinogen- or virus-induced DNA amplification (Schlehofer et al. 1983; Heilbronn et al. 1985; Schlehofer et al. 1986; Schmitt et al. 1989), and selectively kill "initiated" (e.g., carcinogen-treated) cells (Heilbronn et al. 1984). Autonomously replicating parvoviruses (H-1, MVM) preferentially kill transformed cells (Mousset and Rommelaere 1982; Chen et al. 1986; Cornelis et al. 1988a; Cornelis et al. 1986; Mousset et al. 1986; Faisst et al. 1989; Salomé et al. 1990; Guetta et al. 1990) and their nonstructural proteins are cytotoxic essentially in tumorigenic cells (Cornelis et al. 1988b; Brandenburger et al. 1990; Caillet Fauquet et al. 1990).

Little is known concerning the mechanism of inhibition of tumorigenesis in vivo. Stimulation by parvovirus infection of immunological functions of the host leading to oncosuppression seems not to be very likely: even AAV with large deletions (up to 70%) within its genome was shown to inhibit oncogenicity of adenoviruses (de la Maza and Carter 1981) and there is no simple correlation between parvovirus H-1 titer and inhibition of tumors induced by 7,12-dimethylbenz(a)anthracene in hamsters (Toolan et al. 1982).

Aside from its antiviral action interferon (IFN) is known to act cytostatically on some tumor cells (for review see Clemens and McNurlan 1985). Viruses might contribute to the inhibition of cell transformation by induction of IFN in their host cells. However, as far as parvoviruses have been studied, they proved to be inefficient inducers of IFN in vivo (Kilham et al. 1968; Harris et al, 1974; Darrigrand et al. 1984; Wiedbrauk et al. 1986a, b). Furthermore, the anticarcinogenic activity of AAV in vivo could not be achieved by poly(dI-dC) (de la Maza and Carter 1981), which is known to efficiently induce IFN.

Growth inhibition of some carcinoma cells also has been shown by the biological response modifier interleukin-6 [IL-6; synonym: IFN-β2; (Chen et al. 1988)]. It can be induced by virus infection and by other cytokines, e.g., tumor necrosis factor (TNF; Kohase et al. 1986; Le and Vilcek 1989; Sehgal et al. 1989) and plays an important role in immune response regulation (Sehgal et al. 1989). IL-6 acts synergistically with TNF and IL-1 and is involved in T cell activation (Le and Vilcek 1989; Billiau et al. 1991).

TNF-α is another potent pleiotropic response modifier (Old 1985; Beutler and Cerami 1989) in inflammatory and immunological host defense reacions. It was first discovered by its antitumor activity in mice (Carswell et al. 1975). TNF enhances the cytotoxic activity of NK cells and macrophages and causes necrosis and regression of some solid tumors in vivo. Therefore it has been used as an anticancer agent. TNF can be induced by different types of infectious agents, i.e., bacteria and viruses (Carswell et al. 1975; Berent et al. 1986; Aderka et al. 1986; Lorence et al. 1988; Ray et al. 1988). The biological activities of TNF partially overlap with those of IFN, e.g., antiviral activity and growth inhibitory effects. These effects are probably mediated by IFN-β (Jacobsen et al. 1989).

Methods and results

To assess in vitro a possible role of IFN in parvovirus-mediated suppression of cell transformation and tumorigenesis, we tested the effect of infection by parvoviruses on the murine IFN-β promoter transfected into human (HEp-2) and murine (C243) culture cells as a very sensitive indicator system (Ohno and Taniguchi 1983; Zinn et al. 1983; Maroteaux et al. 1983; Fujita et al. 1985; Goodburn et al. 1985; Goodburn et al. 1986; Dinter and Hauser 1987; and see below). The parvoviruses AAV types 2 and 5, H-1, MVM (prototype strain), adenovirus type 2, and Newcastle disease virus (NDV, for control purposes) were used in the experiments.

To establish cellular clones containing an inducible IFN-β promoter, an *Eco*RI/*Pst*I fragment of the murine IFN-β locus spanning approximately −1300 to +6 relative to the transcriptional start site and, thus, containing all 5′ upstream sequences necessary for virus induction was cloned into pBlueluc yielding pBluelucβ15. The same fragment was also cloned into pSVM-1 leading to plasmid pSVMβ1. The human IL-6 promoter (a kind gift of Dr. W. Fiers) was cloned as a 1200-bp *Bam*H1/*Xho*I fragment into pSVM-1. pSVM-1 contains the *E. coli* β-gal gene, and pBlueluc contains the firefly luciferase gene. Both genes are fused to the SV40 t intron and the polyadenylation site cloned into the pBluescript KSM13+ vector (Stratagene, Heidelberg, Germany). Transfection experiments were carried out according to the method of Chen and Okayama (Chen and Okayama 1987) with minor modifications.

From C243 murine fibroblasts [transformed by Moloney sarcoma virus; high IFN-producing capacity; (Oie et al. 1972)] that were cotransfected with pBluelucβ15 and pSV2Neo (Southern and Berg 1982) and selected with G418 (BRL, Berlin, Germany), we obtained clone Muβ15. C243 cells cotransfected with pBluelucβ15, pSVMIL6 and pSV2Neo resulted in clone IL6β15. HEp-2 (human laryngeal carcinoma cell line) cells were cotransfected with pBluelucβ15 and the construct pSV2Pac, conferring puromycin resistance (Vara et al. 1985; 1986). After selection with puromycin, clone Hep 1300Luc was isolated.

As a specificity control, both parental cell lines (C243, HEp-2) were also transfected transiently and stably with pSVM1, pBlueluc and pSVMβ1. In contrast to pSVM1, pBlueluc showed a low, but significant signal over background which could be reduced to background level by cloning either the murine IFN-β promotor or the SV40 polyadenylation site in front of the luciferase gene. These results indicated that a small number of transcripts originated from the pBluescript vector.

To test for induction of luciferase activity from the IFN-β promoter, Muβ15 cells, IL6β15 cells, and Hep1300Luc were seeded into 12- or 24-well dishes and infected with AAV type 2 [heated to 60°C for 30 min to inactivate adenovirus (used for propagation of AAV)], with AAV types 2 or 5 (CsCl-purified viruses), H-1, adenovirus type 2 (ad-2), and Newcastle disease virus (NDV), or treated with TNF (500 ng/ml). NDV infection or treatment with TNF was used as "positive" controls (Lorence et al. 1988; Ray et al. 1988) and adenovirus infection (with live or heat-inactivated virus) was performed to analyze any effects of adenovirus in experiments with the heat-"inactivated" AAV type 2. After incubation overnight, cells were inoculated with virus at the multiplicities of infection (m.o.i.) indicated in Fig. 1 and incubated for 1 or 2 more days. At 24 or 48 h, cells were harvested for determination of luciferase induction (for details see legend to Fig. 1). After adjusting for protein concentrations of the cell extracts, luciferin was mixed with

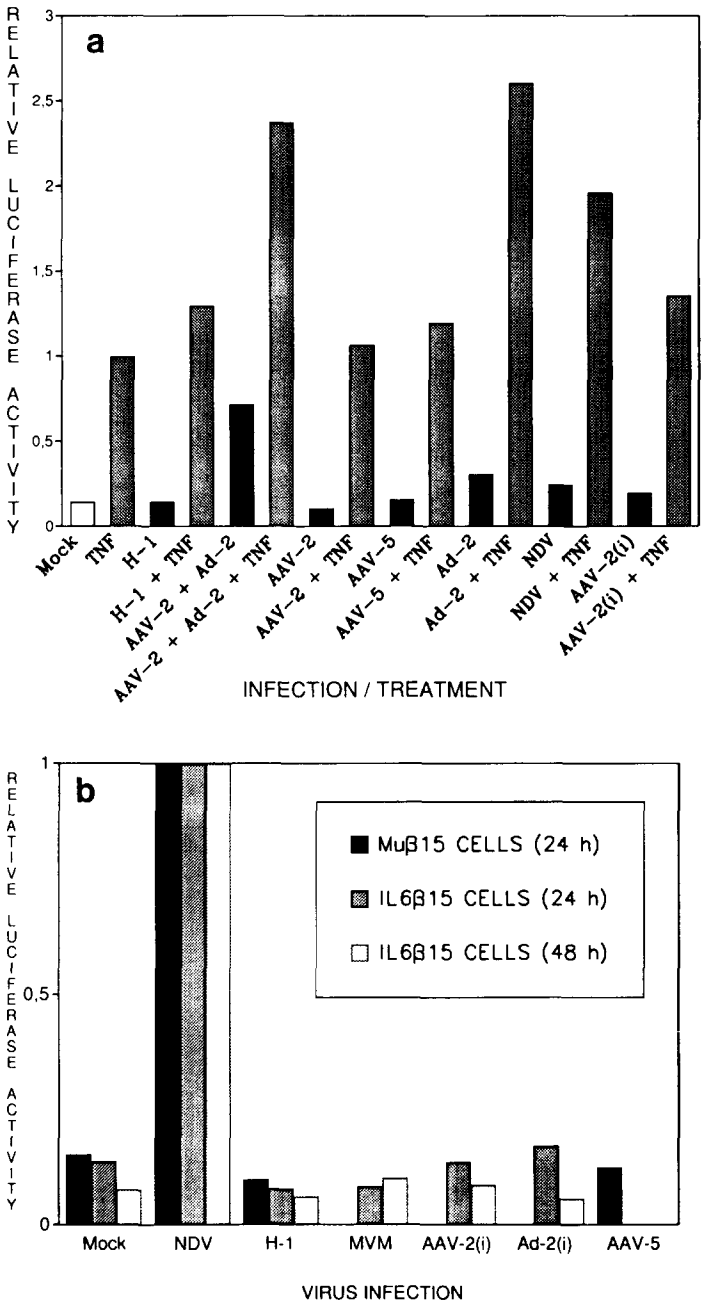

Fig. 1a, b. Relative luciferase activity from the interferon-β (IFN-β) promoter after parvovirus infection of human and murine cells. Cells containing the luciferase gene under the control of the IFN-β promoter [Hep1300Luc **(a)** and Muβ15, IL6β15 **(b)**, see text] were grown on plastic in Eagle's minimal essential medium (Dulbecco's modification; Biochrom, Berlin, Germany) supplementd with 10% fetal calf serum (Gibco, Eggenstein, Germany) and antibiotics. Cells were incubated at 37° C in a humidified atmosphere (5% CO_2). For determination of luciferase activity from the IFN-β promoter, cells were seeded into 12- or 24- well dishes (Greiner, Nürtingen, Ger-

extracts and assayed for induction of luciferase. As shown in Fig. 1 (a, b), none of the parvoviruses, AAV types 2 and 5, H-1 or MVM induced luciferase production from the IFN-β promoter. This is in contrast to infection with NDV (notably in the murine cells), with adenovirus type 2, or to treatment with TNF (positive controls). Figure 1 a demonstrates in addition that NDV or adenovirus infection can enhance TNF-induced stimulation from the IFN-β promoter, whereas infection with AAV or H-1 does not influence the level of luciferase induced by TNF. Similarly, the β-galactosidase reporter gene driven by the IFN-β promoter was not induced in cells infected with parvoviruses (data not shown).

There were several reasons for measuring transient expression of luciferase activity using the IFN-β promoter to determine the capability of induction of IFN-β by parvoviruses. First, the luciferase protein was reported to have a very short half-life in vivo because it contains a peroxisomal targeting sequence (Keller et al. 1987). Second, biological half-life analysis showed that luciferase is degraded more rapidly than other indicator proteins, e.g., chloramphenicol acetyl transferase (CAT) or β-galactosidase (β-gal) (Thompson et al. 1991; cf., Brasier et al. 1989). Third, a kinetics analysis of TNF-induced stimulation of luciferase in Hep-2 cells revealed that its maximal expression takes place 6 h after treatment decreasing to 50% 6 h later (M. Rentrop, unpublished results). On the other hand, "natural" IFN-β protein is very stable in culture medium and accumulates in contrast to transient luciferase activities as measured in our system. These facts render transient assays more reliable when determining induction of IFN-β. The extremely high sensitivity in detecting induction of the IFN-β promoter in the system used here was recently demonstrated by Jacobsen et al. (1989) who showed that luciferase activity induced by TNF via the IFN-β promoter was detectable at conditions where IFN-β mRNA was detectable only by polymerase chain reaction analysis.

For control purposes, we tested the sensitivity of parvoviruses to the antiviral effect of IFN in L929 cells (for MVM infection) and HeLa cells (for H-1 infection),

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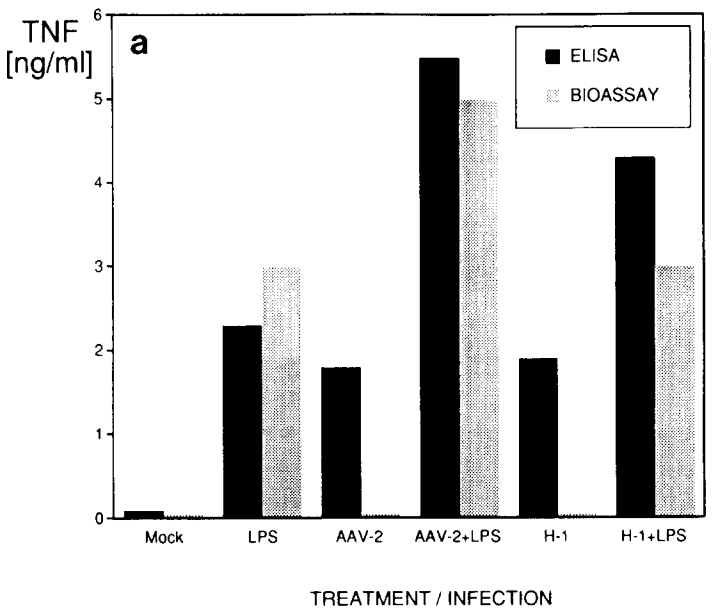
many; 1×10^4 or 3×10^4 cells per well, respectively). After incubation overnight, PBS-washed cells were inoculated with virus [AAV types 2 or 5 (CsCl-purified): 100 $TCID_{50}$ units/cell; AAV type 2 (heated to 60°C for 30 min to inactivate adenovirus used for propagation of the helper virus-dependent AAV; "AAV-2(i)": 10 $TCID_{50}$ units/cell; H-1: 10 pfu/cell; MVM: 10 pfu/cell; adenovirus type 2: 1 pfu/cell]; NDV 0.1 H.U./cell, or treated with TNF (500 ng/ml). NDV infection or treatment with TNF were used as "positive" controls for C243 or Hep-2 cells, respectively. Adenovirus infection [with live or heat-inactivated virus (as above) "Ad-2(i)"] was performed to analyze effects of adenovirus in experiments with heat-"inactivated" AAV type 2 ["AAV-2(i)"]. After adsorption at 37°C for 1 h medium was replenished and cells were incubated (37°C; 5% CO_2) for 1 or 2 more days. At 24 or 48 h after infection, cells were washed with PBS and removed using a cell scraper. Cells were then pelletted and chilled to −70°C followed by two more rounds of freezing and thawing. Cell extracts were suspended in 250 mM TRIS/HCl (pH 7.8), mixed thoroughly and spun down (low-speed centrifugation). Supernatants were analyzed for the concentration of protein using the Bio-Rad protein assay (Bio-Rad, München, Germany). After adjusting the protein concentrations of the cell extracts to a bovine serum albumin standard, 250 µl of Luciferin [Boehringer, Mannheim, Germany; 0.25 mM in 15 mM $MgCl_2$, 25 mM TRIS/HCl (pH 7.8), 5 mM ATP]) was mixed with extracts and assayed for induction of luciferase in a "Lumat" analyzer (Berthold, Wildbad, Germany). **a** Relative induction of luciferase in HEp1300Luc cells, 24 h after infection. Luciferase activity as induced by TNF is set as 1. Columns represent means of triplicate experiments. **b** Relative induction of luciferase in Muβ15 or IL6β15 cells, 24 or 48 h after infection, as indicated. The induction level of transcription from the IFN-β promoter by NDV infection is set as 1



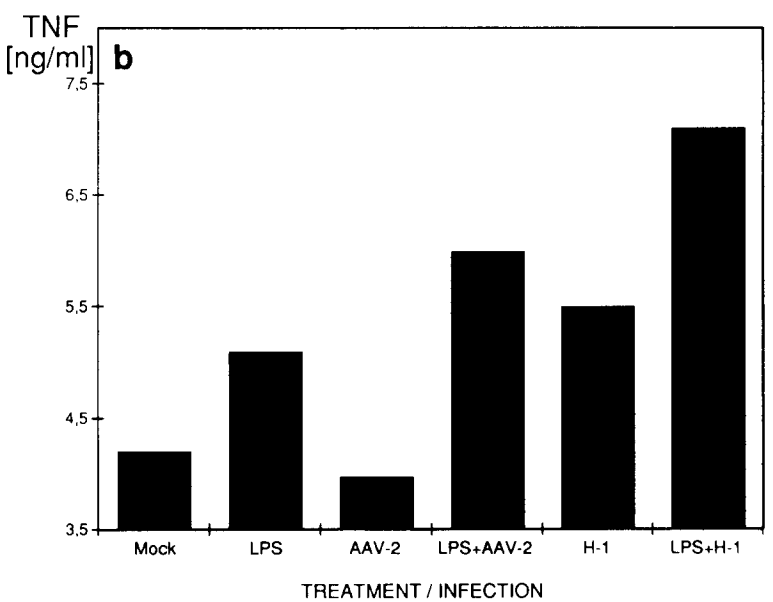


Fig. 2a, b. Analysis of induction of tumor necrosis factor-α (TNF-α) in THP-1 or RAW264 cells after parvovirus infection. THP-1 cells grown in RPMI1640, 10% fetal calf serum **(a)** or RAW264 cells grown in D-MEM, 10% fetal calf serum **(b)** were infected with AAV or H-1 or treated with lipopolysaccharide (LPS, 10 g/ml; from *Salmonella Minnesota;* Sigma, Geisenhofen, Germany), respectively, and tested for induction of TNF. Cells in plastic dishes (RAW264: $2.5 \times 10^5/9cm^2$; THP-1: 1×10^6/ml) were infected or treated, respectively. In some experiments cells were additionally treated with LPS 1 h prior to ("LPS + AAV-2 or H-1") or after ("AAV-2 or H-1 + LPS") infection. Cells were washed with PBS and inoculated with purified virus (cf. Fig. 1;

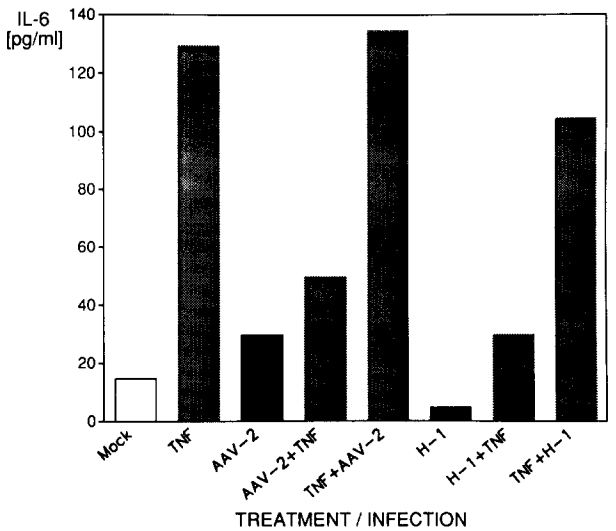


Fig. 3. Analysis of induction of interleukin-6 (IL-6) by parvovirus infection in HeLa cells. HeLa cells (2.5×10^5/9 cm^2 well of a six- well plastic dish) were infected with AAV type 2 or H-1 (m.o.i.'s in Fig. 1 and 2, respectively) or treated with TNF [500 ng/ml (similar results with 2 g/ml)] and assayed (24 h after infection) for the presence of IL-6 in the supernatant. In some experiments cells were additionally treated with TNF 1 prior to ("TNF + AAV-2 or H-1") or after ("AAV-2 or H-1 + TNF") infection. IL-6 concentration was determined using the InterTest-6X Human IL-6 ELISA (Genzyme, Cambridge, Mass., USA)

respectively. Cells were treated with 1000 IU/ml or 100 IU/ml of murine (L929 cells) or human (HeLa cells) IFN-α/β or murine IFN-γ, or mock-treated. After 18 h, cells were inoculated with MVM (10 to 0.0001 pfu/cell) or H-1 (100 to 0.0001 pfu/cell). Uninfected cells or cells infected with vesicular stomatitis virus (VSV) were used as negative and positive controls, respectively. Cells were checked for cytopathic effects 3 or 4 days after infection. As expected, parvoviruses proved to be sensitive to IFN-α or γ [revealed by an inhibition of the cytopathic effect (CPE) of MVM infection in L929 cells and of that of H-1 infection in HeLa cells (data not shown)]. These observations are in line with the results of others reporting sensitivity of parvovirus MVM to IFN (e.g., Harris et al. 1974).

In additional experiments we used THP-1, HeLa or RAW 264 cells, to test for an induction of TNF or of IL-6 after infection with AAV or H-1, respectively. As shown in Fig. 2, parvovirus infection (AAV type 2; H -1) led to a limited induction of TNF in THP-1 (Fig. 2a) or RAW264 (Fig. 2b) cells, similar to the stimulation with lipopolysaccharide (LPS; positive control) as measured by ELISA. In

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adsorption for 30 min at 37° C) at an m.o.i. of 50 pfu/cell (AAV type 2), of 500 pfu/cell (H-1, AAV type 5). Uninfected or LPS-treated cells were used as controls. After 16 h, supernatant of the cells was removed and (after low-speed centrifugation) analyzed for the presence of TNF. Induction of TNF was measured by analysis of the concentration of TNF in the supernatant of treated or infected cells using ELISA **(a)**. Analyses by bioassay [cytotoxicity on murine fibroblasts as described by Männel & Falk (Männel and Falk, 1989)] are presented in **a** and **b**. (Mouse TNF could only be measured by bioassay)

bioassays, however, no induction of TNF by parvovirus infection was found in THP-1 cells, whereas a slight enhancement of the level of LPS-induced TNF synthesis could be observed (Fig. 2a, b). Similar results were obtained in HeLa, HL60 and U937 cells (data not shown). In parallel to the absence of IFN-β induction, parvovirus infection also failed to induce synthesis of IL-6 in HeLa (Fig. 3) or THP-1 cells (not shown). In some experiments, however, a slight increase of TNF (positive control)-induced IL-6 synthesis was observed.

Discussion

The tumor-suppressive effects of parvovirus infections on a variety of tumors at different sites and of different origin (i.e., irrespective of the mode of tumor induction and of the transforming agent), suggest a rather unspecific action of viral functions on tumor cells. Since a general stimulation by parvovirus infection of the host's immune system seems unlikely (cf. references cited above), we tested in assays in vitro whether parvovirus infections would possibly lead to the induction of IFN or other physiological response modifiers to many virus infections, known to interfere also with tumor cell growth (cf., Clemens and McNurlan 1985; Sehgal et al. 1989; Billiau et al. 1991; Loetscher et al. 1991). We were able to show in very sensitive cell culture assays (measuring the induction of the IFN-β promoter) that neither autonomously replicating parvoviruses (MVM, H-1) nor AAV induced IFN-β in rodent or human cells; since factors or agents inducing the expression of IFN-α also induce IFN-β it is conceivable that IFN-α is likewise not induced by parvovirus infection. This is in line with the findings of a weak IFN induction in vivo. Although parvovirus MVM has been reported to induce IFN in infected mice (Harris et al. 1974), the levels of IFN induced were relatively low [IFN was measured by the yield reduction of mouse polio virus (strain GD-7) HA in L929 cells]. The low levels of IFN seen with MVM, a single-stranded DNA virus, were in contrast with those observed with the single-stranded RNA virus, EMC (Harris et al. 1974). In the same study, MVM was shown to be very sensitive to the (virus-) inhibiting action of IFN.

These results are consistent with the study of Kilham et al. (1968) in which Kilham rat virus (KRV) was shown to induce only very low levels of IFN as detected in serum specimens from infected rats. Similar results were reported by Darrigrand et al. (1984) showing that KRV did not induce measurable serum IFN in infected rats. Also in these studies, KRV was sensitive to the antiviral effect of IFN. The mink parvoviruses, Aleutian disease virus (ADV) and mink enteritis virus (MEV) neither induced nor were sensitive to the effects of IFN (Wiedbrauk et al. 1986). Furthermore, these authors reported that preexisting parvovirus infections did not inhibit poly(I) poly(C)-induced IFN production.

MVM was found to inhibit various functions mediated by murine T cells in vitro. These included inhibition of lymphocyte proliferation and generation of cytolytic T lymphocyte activity but not IFN-α or γ induction (Engers et al. 1981). However, from some of their results, the authors suggest that pretreating cell cultures with exogenous murine IFN-α or γ does not inhibit the replication of MVM.

It might be possible that the known influences of parvovirus infection on the cell cycle (Winocour et al. 1988; Klein-Bauernschmitt et al. 1992) are involved in

the inefficient induction of cytokine synthesis under the conditions described herein.

In our study, lytically replicating parvoviruses (MVM, H-1) were demonstrated to be sensitive to the antiviral action of IFN-α or γ in human and murine cell lines. This sensitivity of MVM to the antiviral effects of IFN has been observed also by others (Harris et al. 1974). However, these results are at variance with the report by Engers et al. (1981).

Our results demonstrate in addition that parvoviruses may be weak inducers of the cytokines TNF or IL-6 in the cell cultures tested. In some experiments, a slight increase of the expression of these cytokines was observed when cells treated with potent inducers were additionally infected with parvoviruses. Thus, it is conceivable that parvovirus infection "assists" in the synthesis of cytokines.

The results reported herein do not definitely exclude that induction of IFN might play some role in parvovirus-mediated tumor suppression in vivo. However, the apparent inability of the parvoviruses used in this study to induce detectable levels of IFN in vitro as well as the very weak induction observed in vivo make this unlikely.

Further in vivo investigations might well clarify whether the slight enhancement of synthesis of cytokines when induced by other agents could contribute to the rather unspecific tumor-suppressive properties of parvoviruses. This study does not reveal mechanisms by which parvoviruses can act as antitumor agents. Our findings make it rather unlikely that an induction of cytokines plays an important role in the observed tumor-suppressive properties of these agents.

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