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Cytokine regulation of lactate dehydrogenase-elevating virus: inhibition of viral replication by interferon-γ

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Summary

The mechanisms which regulate the replication of lactate dehydrogenase-elevating virus (LDV), a persistent murine model virus which infects macrophages, are unclear. For this study, the effects of murine recombinant interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) on LDV replication were examined. LDV permissiveness was reduced in macrophages obtained from uninfected mice treated with IFN-γ prior to cell harvest and in vitro LDV infection. Virus inhibition by IFN-γ was also observed when neonatal LDV-infected mice were injected with this cytokine prior to macrophage harvest and analysis of LDV replication-positive cells. Persistently LDV-infected mice demonstrated an increase in viremia levels following treatment with TNF-α. Neither IFN-γ nor TNF-α had any direct in vitro effect on LDV replication in cultured macrophages, suggesting that the actions of these cytokines required secondary or accessory in vivo events. These results provide evidence for cytokine-mediated regulation of LDV infection and support a role for the immune system in the LDV-host relationship.

IFN-γ; TNF-α; Lactate dehydrogenase-elevating virus

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Introduction

Lactate dehydrogenase-elevating virus (LDV, reviewed in Rowson and Mahy, 1985; Cafruny, 1989) causes a persistent infection of mice, during which the virus replicates in a subpopulation of macrophages and survives in the blood as an immune complex with specific IgG antibodies (Cafruny and Plagemann, 1982a). LDV has a single-stranded RNA genome which resembles that of equine arteritis virus (Godeny et al., 1990), and has been recently included in a new group of positive-strand viruses (Plagemann and Moennig, 1992). LDV is the agent of fatal paralytic disease in genetically susceptible mice (Martinez et al., 1980), and has been widely studied as a model virus for various aspects of the virus-host relationship (Rowson and Mahy, 1985; Cafruny, 1989).

Data concerning the role of cytokines in LDV infection are scanty, although a transient interferon-α/β response to LDV infection has been demonstrated (Evans and Riley, 1968; Dubuy et al., 1973; Lussenhop et al., 1982), LDV stimulates interferon-α/β in cultured macrophages (Lagwinska et al., 1975), and increased amounts of interferon-γ were extracted from spleens of LDV-infected mice at 24 h post-infection (Bradley et al., 1991). In the present report, we have investigated the effects of purified recombinant murine interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) on LDV replication in macrophages and on viremia. These cytokines have important immunoregulatory and anti-viral activities (Pestka and Langer, 1987; Wong et al., 1992; Wong and Goeddel, 1986), and play a key role in host defense, for example through the induction of MHC class I and II cell surface proteins, and of inflammatory responses (Farrar and Schreiber, 1993). Despite these potential anti-viral effects, their role in persistent viral infections is obscure since there is a failure of virus clearance, and thus an examination of cytokine effects on LDV infection is of interest.

Materials and Methods

Viruses. Virus stocks of the LDV-P strain (Cafruny and Plagemann, 1982b) were prepared from the 24 h post-infection (p.i.) plasma of outbred CF1 mice and stored at $-80°C$. Virus titrations were determined by injecting serial 10-fold dilutions of mouse plasma into uninfected indicator mice, and assaying blood LDH at 4 days post-injection for the increase which is indicative of LDV infection (Plagemann et al., 1963).

Mice. CF1 outbred mice were obtained from Sasco (Omaha, NE) and maintained in the USD Animal Care Facility.

Cytokines and antibodies. Purified recombinant murine TNF-α (lot no. M3-RD55; 1×10^8 u/mg), TNF-β, IFN-γ (lot no. M3-RD48; 5×10^7 u/mg),

leukemia inhibitory factor (LIF), transforming growth factor-β (TGF-β), and interleukin-1 (IL-1) were provided by Genentech, Inc. All cytokines displayed only a single band by polyacrylamide gel electrophoresis and contained <2 endotoxin units/ml. Mice were injected with cytokine diluted in phosphate-buffered saline (PBS, pH 7.4) containing 0.5% bovine serum albumin (BSA). Cytokine dosages were based on previous results demonstrating in vitro (Wong and Goeddel, 1986; Koyanagi et al., 1988) or in vivo (Eddy et al., 1992; Beck et al., 1991) actions of TNF and IFN-γ on cultured cells or in animals, respectively. IFN-γ and TNF-α were given either intraperitoneally or intravenously in doses of 4–5 μg, or were added to macrophage cultures at 5–10 μg/3 ml of medium. Monoclonal hamster anti-murine TNF-α antibodies (TN3–19; Sheehan et al., 1989) were provided by Genentech, Inc.

In vitro LDV infection. Primary cultures of peritoneal macrophages were established in minimum essential medium containing 5% fetal calf serum (Stueckemann et al., 1982b). For assessment of in vivo cytokine effects, adherent macrophages were derived from cells obtained by peritoneal lavage of mice with 1–4 ml PBS. Macrophage cultures from uninfected mice were exposed to an optimum multiplicity of LDV (approximately 500–1000 ID_{50}/cell) for 60 min, followed by addition of fresh culture medium (Stueckemann et al., 1982b). Macrophage cultures from mice previously LDV-infected were acetone-fixed between 3–4 h after removal from the animal and adherence on glass coverslips.

Fluorescence assay for LDV infection. Macrophage cultures LDV-infected in vitro for 8 h, or obtained from LDV-infected neonatal mice, were fixed in acetone, and the cells which contained replicating LDV were identified by fluorescence immunoassay as described previously (Cafruny et al., 1986). Briefly, fixed cells were overlayed with murine polyclonal IgG anti-LDV

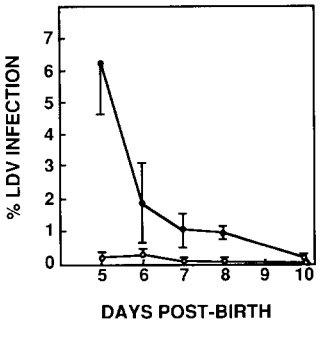

Fig. 1. Legend: Mice were LDV-infected within 24 h of birth, and perotoneal macrophages were obtained between 5–10 days of age (x-axis). Mice received 2 μg IFN-γ (0) or PBS-0.5% BSA as a control (•) 18 h prior to peritoneal cell harvest. LDV-positive cells were determined by FA analysis. Bars indicate standard deviations for 2–4 determinations.

antibodies for 1 h, followed by FITC-conjugated anti-mouse IgG (Southern Biotech). Fluorescent (LDV-infected) cells were enumerated in a fluorescence microscope, counting between 200–800 cells/coverslip. For determination of the percentage of LDV-infected cells from IFN-γ-treated neonates (Fig. 1), the entire coverslip culture was scanned for positive cells, and the total number of cells was estimated from representative field counts.

Statistical analysis. The significance of the difference between mean percentages was determined by calculating the t statistic using the arcsin transformation (Sokal and Rohlf, 1968).

TABLE 1

Effects of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TFN-α) on LDV replication

Expt.	Cytokine	% Macrophages LDV-infected		% Change
		T-0	T-18	
(A) Interferon-γ				
1	IFN-γ	11	2	−82
	–	35	34	−3
2	IFN-γ	10	4	−60
	–	15	15	0
3	IFN-γ	8	5	−38
	–	6	7	+17
4	IFN-γ	10	8	−20
	–	22	17	−23
5	IFN-γ	36	2	−94
	IFN-γ	32	12	−62
	–	19.5	15	−24
	–	27	7	−74
6	IFN-γ	18	4	−78
	IFN-γ	8	1	−88
	–	12	11	−8
	–	12	14	+17
			Mean IFN-γ response =	−65% ± 26%
			Mean control response =	−12% ± 29%
				t = 3.7178
				$P<0.01$
(B) Tumor necrosis factor-α				
7	TNF-α	35	32	−8.6
8	TNF-α	15	9	−40
9	TNF-α	30	23	−23
10	TNF-α	28	28	0
11	TNF-α	16	13	−19
12	TNF-α	27	20	−26
13	TNF-α	19	19	0
			mean TNF-α response =	−16.7% ± 15%

Mice were outbred CF1, approx. 5–7 weeks old.

Mice were injected with 4 μg IFN-γ, TNF-α or PBS as control (−). Peritoneal macrophages were sampled at times 0 and 18 h post-injection, infected with an optimal multiplicity of LDV, and stained by immunofluorescence for the % of macrophages replicating the virus.

Results

Interferon-γ (IFN-γ) suppresses macrophage LDV permissiveness

The effects of murine IFN-γ and TNF-α on macrophage LDV permissiveness were studied after in vivo cytokine administration. Peritoneal cells were obtained from individual mice before, and 18 h after, the injection of IFN-γ or TNF-α, cultured on glass coverslips, and the adherent macrophages were infected with an optimum multiplicity (500–1000 ID_{50}/cell) of LDV. Macrophage LDV permissiveness was assessed by fluorescence antibody (FA) analysis of virus-exposed cells (Cafruny et al., 1986). Table 1 shows the results of these experiments, in which the control (T-O) macrophage permissiveness for LDV ranged between 6–36%, consistent with typically observed analyses of individual mice (Stueckemann et al., 1982b; Plagemann and Moennig, 1992). FA analysis at 18 h. post-cytokine treatment demonstrated a significant suppression of LDV-permissiveness in macrophages from IFN-γ-treated mice (Table 1). Although the IFN-γ and TNF-α injections were about equally distributed between the i.v. and i.p. routes, either route of administration yielded similar results. The mean suppression observed in macrophages from 8 IFN-γ-treated mice was 65% vs. 12% suppression in control (PBS/BSA-injected) experiments ($P<0.01$). In contrast to the suppressive action of IFN-γ, the data in Table 1 show that TNF-α failed to significantly depress macrophage LDV permissiveness under identical experimental conditions (mean suppression from 7 experiments = 16.7%).

When cultured macrophages from untreated normal mice were exposed in vitro to IFN-γ, TNF-α, or IFN-γ + TNF-α combined, there was no effect on subsequent LDV permissiveness (Table 2), indicating that the suppressive action of IFN-γ on the macrophage response to LDV was indirect. In other experiments (data not shown), in vitro addition of the same dose of IFN-γ protected same-strain mouse macrophages from destruction by *Mycobacterium*

TABLE 2

Effects of TFN-α, IFN-γ, or TFN-α + IFN-γ on macrophage cultures following direct addition in vitro

[1]Cytokine Treatment	Expt	% of Macrophages LDV-infected	
		Control	Cytokine(s)
TNF-α	1	3.9%	3.3%
	2	36.0%	33.5%
	3	33.5%	33.0%
IFN-γ	1	3.9%	2.9%
	2	36.0%	32.5%
	3	33.5%	30.5%
TNF-α + IFN-γ	1	3.9%	3.7%

[1]TNF-α (5–10 μg), IFN-γ (5–10 μg), or TNF-α + IFN-γ (5 μg each) were added to macrophage cultures 18 h prior to LDV infection with an optimal multiplicity of virus (500–1000 ID_{50}/cell).

smegmatis, demonstrating in vitro efficacy of this cytokine in the induction of macrophage defense against bacterial infection.

Effect of IFN-γ on neonatal mouse macrophage LDV replication

Macrophages from chronically LDV-infected adult mice display practically undetectable levels of LDV replication, due to exhaustion of LDV permissive cells (Stueckemann et al., 1982b). However, macrophages from uninfected newborn mice (1–2 week-old) display elevated LDV permissiveness when infected in vitro (Onyekaba et al., 1989a). Therefore, we examined LDV replication in macrophages obtained from control and IFN-γ-treated neonatal mice which had been LDV-infected during the first 24 h post-birth. Consistent with previous observations, Fig. 1 shows that macrophages from 5–8 day old mice even though infected at birth, contained significant (0.6–7.4%) LDV permissiveness, thus providing a background upon which to examine the effect of IFN-γ. As shown in Fig. 1, pretreatment of LDV-infected neonatal mice with 2 μg of IFN-γ administered i.p. 18 h prior to cell harvest reduced the replication of endogenous LDV to the essentially negligible levels as seen in chronically-infected adult mice (0.0–0.2%; Stueckemann et al., 1982b). By 10 days of age, the levels of LDV replication in control mice had fallen to similar levels (0.04–0.10%, Fig. 1), but even these levels were reduced further by IFN-γ. Thus, the in vivo effect of IFN-γ on LDV replication was confirmed by the study of highly LDV-permissive neonatal mice. Attempts to examine any effect of IFN-γ on viremia in these mice failed to demonstrate a difference due to prior cytokine treatment (data not shown), but this failure might be attributed to the lack of sensitivity of our assay procedures which could not detect less than about a 0.5 $\log_{10}$ change.

Tumor necrosis factor-α (TNF-α) enhances chronic viremia levels in LDV-infected mice

Chronically (2–4 month p.i.) LDV-infected mice received TNF-α or IFN-γ injections by the intravenous (i.v.) or intraperitoneal (i.p.) routes. Blood samples were obtained from the mice immediately prior to, and 24 h after, cytokine administration, and blood plasma LDV titers were determined. Table 3 shows the results of all nine experiments carried out, in which treatment of chronically LDV-infected mice with TNF-α stimulated the elevation of plasma LDV levels, from a mean of $10^{5.4}$ ID_{50}/ml to a mean of $10^{6.7}$ ID_{50}/ml 24 h after cytokine treatment. There was no significant change in viremia associated with control injections of PBS or with IFN-γ treatment (Table 3). The effect of TNF-α was found to be abrogated by prior administration of a monoclonal anti-TNF-α antibody (Table 3), showing that the TNF-α response could be inhibited in vivo.

Lack of cytokine effect on LDV infection during low-dose virus challenge

Uninfected mice were given TNF-α, IFN-γ, or TNF-α + IFN-γ in combination, 18 h prior to exposure to a low-dose challenge of LDV (< 100 virions/

TABLE 3

Effects of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) on viremia in chronically LDV-infected mice

Expt.	TNF-α-Treated		Control		IFN-γ-Treated		TNF-α & Anti-TNF Treated	
	T-0	T-24	T-0	T-24	T-0	T-24	T-0	T-24
1	5.5	7.0	5.0	5.5	6.0	6.5	5.5	5.5
2	7.0	7.0	5.5	5.5	5.5	5.5	5.0	5.5
3	5.5	6.5	5.5	6.5	5.5	6.0	6.0	6.5
4	5.0	6.0	5.5	5.0			4.0	4.5
5	6.0	7.0	≤4.0	≤4.0			5.5	5.5
6	5.5	6.0	5.0	5.0			5.5	5.0
7	≤4.0	6.5	5.5	5.0				
8	6.0	7.0	5.0	≤4.0				
9	≤4.0	7.5	5.0	5.0				
mean =	5.4±0.96	6.7±0.51	5.1±0.49	5.0±0.75	5.67±0.3	6.0±0.5	5.25±0.69	5.4±0.67

Chronically LDV-infected (2–4 months p.i.) young adult outbred CF-1 mice received 5 μg TNF-α, 5 μg IFN-γ, or PBS (control), given i.v. or i.p. at $T=0$ h. Mice were bled at $T=0$ h and $T=24$ h. Blood plasma was assayed for virus titer, expressed in the Table as $\log_{10}ID_{50}$/ml. In some experiments, mice were injected with monoclonal anti-TNF-α given in two 100 μg doses at 1 and 2 h prior to TNF-α injection.

mouse). There was no protective effect of any of these cytokine treatments, since the majority of mice became infected in all cases: TNF-α (8:9 infected); IFN-γ (9:10 infected); TNF-α + IFN-γ (6:7 infected); PBS/BSA control (10:10 infected). Thus, no protective role in LDV-naive mice can be ascribed to these cytokines.

Effects of other cytokines on LDV infection

A panel of four additional recombinant murine cytokines (LIF, TGF-β, IL-1, and TNF-β) was screened for effects on LDV replication in vivo and in vitro. No significant effects of these cytokines were observed on chronic viremia levels or on LDV permissiveness of macrophages obtained from mice treated with these cytokines (Table 4). Furthermore, various combinations of these cytokines failed to demonstrate activity on macrophage LDV permissiveness (Table 4). These results support the specificity of TNF-α and IFN-γ in their effects on the LDV response.

Discussion

Previous studies in which the interferon (IFN-α/β) response of mice to LDV infection was measured demonstrated a transient increase in blood interferon levels between about 1–3 days p.i. (Evans and Riley, 1968; Dubuy et al., 1973; Lussenhop et al., 1982; Plagemann and Moennig, 1992). More recently, the spleens of LDV-infected mice were found to contain increased levels of IFN-γ at 24 h p.i. (Bradley et al., 1991), and cultured spleen cells from 5–7 day LDV-

TABLE 4

Lack of effect of LIF, TGF-β, IL-1, and TNF-β on LDV replication and viremia

(A) Effect on chronic viremia Cytokine	Plasma LDV concentration	
	T-0	*T*-24 h.
LIF	5; 5.5	5.5; 5
TNF-β	5; 5	5; 5
IL-1	4; 5	4; 4
IFN-γ	5; 5; 5; 5	6; 5; 5; 5
TGF-β	5.5; 4	4; 4.5

(B) Effects on Macrophage LDV permissiveness Cytokine:	% LDV infected
LIF	20
TNF-β	20
IL-1	25
IFN-γ	14
TGF-β	18
LIF + TNF-β + IL-1 + IFN-γ + TGF-β	18
TNF-β + IL-1 + IFN-γ + TGF-β	14
IL-1 + IFN-γ + TGF-β	17
IFN-γ + TGF-β	13
LIF + TNF-β + IL-1 + IFN-γ	8
LIF + TNF-β + IL-1	19
TNF-β + IL-1 + IFN-γ	13
LIF + IFN-γ + TGF-β	13
TNF-β + IFN-γ + TGF-β	14
Control	23
Control	20

LIF = leukemia inhibitory factor; TGF-β = transforming growth factor-β; IL-1 = interleukin-1.

infected mice were found to produce increased amounts of IFN-γ relative to cultures of spleen cells from uninfected mice (Plagemann and Moennig, 1992). Despite these observations of LDV-induced interferons, their role in LDV infection is unclear. The coincidence of the timing of peak viremia and IFN-α/β has suggested a role for IFN-α/β in terminating virus production (reviewed in Rowson and Mahy, 1985), but as a better understanding of LDV replication evolved (Stueckemann et al., 1982b), it appeared that LDV was relatively insensitive to IFNs, and that the dynamics of IFN production and viremia reflected cytocidal replication of LDV in a subpopulation of permissive macrophages (Stueckemann et al., 1982a), rather than sensitivity of the virus to induced IFN (Plagemann and Moennig, 1992). Furthermore, although the immune response ultimately fails to clear LDV infection, it remains unresolved whether B- and/or T-cell responses limit the virus during persistent infection (Plagemann and Moennig, 1992). B-cell immunity correlates with perinatal virus protection (Broen et al., 1992; Broen and Cafruny, 1993), and immunodeficient SCID mice may have an extended period of peak viremia relative to normal mice (Broen et al., 1982), suggesting a regulatory role for antibodies, but nude mice which fail to produce anti-LDV antibodies appear to

have normal levels of viremia (Onyekaba et al., 1986b).

The present results are the first to show that LDV replication is sensitive to the effects of IFN-γ, and thus suggest that the production of IFN-γ early in LDV infection (Bradley et al., 1991; Plagemann and Moennig, 1992) may play a role in inhibiting LDV replication in vivo. This finding contrasts with the relative insensitivity of LDV replication to other interferon types (IFN-α/β, reviewed in Plagemann and Moennig, 1992). Sensitivity of LDV replication to IFN-γ may also provide an explanation for the range of LDV permissiveness seen in macrophages from mice of different ages or identities (Stueckemann et al., 1982b; Onyekaba et al., 1989a), since endogenous IFN-γ levels may vary with the development of new microbial exposures. It is of interest that the effect of IFN-γ on macrophage LDV infection was only seen when mice were injected with IFN-γ prior to macrophage harvest. Neither IFN-γ, nor the combination of IFN-γ and TNF-α had any direct effect on macrophage LDV-permissiveness. Although we exposed macrophages to relatively high concentrations of IFN-γ and TNF-α, equal to or greater than those reported effective in other cell culture systems, we cannot exclude that dosages different from those we selected might reveal additional effects. TNF-α has been found to synergize with IFN-γ in the suppression of other virus infections (Wong and Goeddel, 1986), but generally when the cytokines exert direct cellular effects. Thus, the LDV response to IFN-γ appears to be due to an indirect mechanism, requiring in vivo intermediate or accessory reactions.

The failure of chronic LDV blood titers to respond to injected IFN-γ (Table 4) contrasts with the IFN-γ effect on macrophage infection, but might be masked by immunoregulatory effects of anti-LDV or by drastic reduction in permissive macrophages at this time in the infection (Stueckemann et al., 1982a). The observation of enhanced LDV viremia following injection of chronically-infected mice with TNF-α provides a second novel cytokine effect on LDV infection. TNF-α has been previously reported to stimulate chronic virus infections (Soike et al., 1989; Wong et al., 1992). Possible explanations for the effect on LDV include premature lysis of LDV-infected cells by TNF-α (Wong et al., 1992) which could release virions into the blood, or a shift in the in vivo differentiation of LDV-permissive macrophages. Circulating IgG anti-LDV levels were unaffected by TNF-α injection of chronically LDV-infected mice (data not shown), so the TNF-α effect was apparently not mediated by suppression of IgG anti-viral immunity. As with IFN-γ, TNF-α had no direct effect on LDV replication in cultured macrophages.

Thus our results support the indirect in vivo interaction of these two cytokines with LDV replication mechanisms, and demonstrate a potential immune pathway for regulation of persistent LDV infection.

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