Modulation of the host microenvironment by a common non-oncolytic mouse virus leads to inhibition of plasmacytoma development through NK cell activation

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Although many cells undergo transformation, few actually develop into tumours, due to successful mechanisms of immunosurveillance. To investigate whether an infectious agent may play a role in this process, the growth of a plasmacytoma was investigated in mice infected by lactate dehydrogenase-elevating virus. Acutely infected animals were significantly protected against tumour development. The mechanisms responsible for this protection were analysed in mice deficient for relevant immune cells or molecules and after in vivo cell depletion. This protection by viral infection correlated with NK cell activation and with IFN-γ production. It might also be related to activation of NK/T-cells, although this remains to be proven formally. Therefore, our results indicated that infections with benign micro-organisms may protect the host against cancer development, through non-specific stimulation of the host’s innate immune system and especially of NK cells.

INTRODUCTION

Immunosurveillance accounts for the successful elimination of transformed cells and prevents most of them from developing into clinical cancer. Although adaptive immune responses, especially cytolytic T-lymphocytes, are heavily involved in antitumoral defences, the role of innate immune cells and their mediators in the early control of tumour development has been emphasized recently (Dunn et al., 2006; Ullrich et al., 2008). Whilst chronic inflammation may enhance cancer growth, cells of the innate immune system and molecules secreted by these cells contribute to tumour immunosurveillance (Bindea et al., 2010). Among those cells, NK cells have been found to participate in the prevention of fibrosarcoma development in normal mice (Smyth et al., 2001). Immunosurveillance has been shown to prevent cancer development through a concurrent effect of IFN-γ and perforin in immunocompetent animals, independently of concomitant infections (Kaplan et al., 1998; Street et al., 2002). Activation of dendritic cells, possibly as a result of tumour cell recognition by NK and NK/T-cells, and of cross-talk between these cell populations, also leads to the initiation of a successful antitumoral response. Both type I and type II IFNs may play a central role in the setting of this cellular interplay (Dunn et al., 2006).

The characteristics of innate immune responses may be influenced by the host microenvironment and especially by infections that may develop concomitantly with these responses. Many studies that have addressed the question of tumour immunosurveillance were performed in experimental models from which micro-organisms were excluded as far as possible. In natural situations, infections occur frequently, mostly with benign viruses or bacteria, which may strongly affect the immune microenvironment of the host. Thus, we decided to analyse how a virus may interfere with the in vivo development of a tumour. Lactate dehydrogenase-elevating virus (LDV), a mouse nidovirus, usually non-pathogenic, but that can deeply modulate the mouse immune microenvironment (Coutelier & Brinton, 2007), was selected as the most appropriate model. In most mouse strains, LDV infects only a restricted subset of non-essential macrophages (Stueckemann et al., 1982). This
limited tropism, that explains the lack of direct pathogenicity of this infection, allows analysis of the indirect pathogenicity resulting from the virally induced immune modulations. In contrast to other viruses, LDV induces a rapid and persistent viraemia. LDV infection is followed by strong alterations of immune responses that are highly reproducible between individual animals. In addition to a bias in T helper lymphocyte differentiation and to a polyclonal activation of B-lymphocytes, LDV is a strong stimulator of innate immune cells (Coutelier & Brinton, 2007). LDV infection is followed by a burst in secretion of pro-inflammatory cytokines, including IFN-γ (Markine-Goriaynoff et al., 2001) and IL-12 (Coutelier et al., 1995). This results in NK cell activation responsible for strong, but transient IFN-γ production (Markine-Goriaynoff et al., 2002). Through interaction with Toll-like receptor 7, LDV also triggers production of type I IFNs by plasmacytoid dendritic cells (Ammann et al., 2009). As a result, LDV infection provides a unique model to analyse the consequences of a viral infection on the course of concomitant diseases, through alterations of the host immune microenvironment. For instance, LDV-induced activation of non-infected macrophages by cytokines that include IFN-γ leads to exacerbated sensitivity to endotoxin shock (Le-Thi-Phuong et al., 2007a; Su et al., 2012) and to enhanced autoantibody pathogenicity, followed by severe anaemia or thrombocytopenia (Meite et al., 2000; Musaji et al., 2004).

Our results indicated that modulation of the mouse immune microenvironment following LDV infection could protect mice against plasmacytoma development through NK cell activation and IFN-γ production.

RESULTS

Effect of LDV infection on plasmacytoma development

To determine the effect of LDV infection on the development of a tumour, plasmacytoma TEPC.1033.C2 cells were inoculated by intraperitoneal (i.p.) injection into BALB/c mice or into animals that had been infected by i.p. injection of ~2 × 10⁷ ID₅₀ (50 % infectious dose) LDV 1 day before. As shown in Fig. 1, uninfected animals developed a tumour that killed eight out of 10 mice in <2 months. In contrast, nine out of 10 infected mice survived tumoral challenge (Fig. 1) and did not show clinical signs of illness. Although kinetics of plasmacytoma development could differ from one experiment to another, a protective effect of acute LDV infection against TEPC.1033.C2 was found in four independent experiments.

Similar experiments were performed with another plasmacytoma, TEPC.15. As for TEPC.1033.C2, acute LDV infection protected mice against tumour development (Fig. 2a). Indeed, whilst seven out of 10 uninfected animals died from cancer development, nine out of 10 infected mice could control plasmacytoma development. Similar results were obtained in three independent experiments. In contrast, no effect of LDV infection was observed on the development of the P815 mastocytoma in DBA/2 mice (Fig. 2b).

LDV is a cytolytic virus that quickly kills infected cells (Stueckemann et al., 1982). To determine whether LDV impairment of plasmacytoma resulted from cytolytic infection, TEPC.1033.C2 cells were cultured in the presence of the virus. As shown in Table 1, LDV did not affect in vitro TEPC.1033.C2 cell growth. A maximum cell number was observed at 1 week without any significant difference between cells cultured with or without virus (P=0.6857). A similar absence of a direct effect of LDV on TEPC.1033.C2 cells was observed in five independent experiments.

Cells involved in LDV-induced prevention of plasmacytoma development

A preventive effect of a virus on tumour development may result from activation of the immune system rather than from direct lysis of infected tumour cells. A putative role of T-lymphocytes was first examined by infecting BALB/c nu/nu mice before administration of tumour cells. The protective effect of LDV infection against TEPC.1033.C2 was the same as that found in immunocompetent animals (Fig. 3a), indicating that it was not mediated by T-lymphocytes. This protective effect of LDV in nude mice was found in two independent experiments.

As NK cells may participate in the anti-tumour immune response and these cells are activated after LDV infection (Markine-Goriaynoff et al., 2002), their role in LDV-induced inhibition of plasmacytoma was checked by treating mice with an anti-asialoganglioside-GM1 (ASGM1) polyclonal antibody. This treatment was shown previously to suppress NK cell responses in LDV-infected mice, although it had no effect on viral replication (Markine-Goriaynoff et al., 2002). ASGM1 completely abrogated the protective effect of LDV infection against TEPC.1033.C2 (Fig. 3b). A
similar suppression of the protection conferred by the virus after in vivo NK cell depletion was observed in two independent experiments. Thus, NK cells, rather than T-lymphocytes, mediated protection against plasmacytoma after LDV infection.

**Molecular mechanisms of LDV-induced prevention of plasmacytoma development**

After LDV infection, NK cell activation results in enhanced lytic activity. However, this increased lytic activity was observed against typical NK cell targets, such as YAC-1 cells, but did not result in enhanced lysis of TEPC.1033.C2 cells (Markine-Goriaynoff et al., 2002). In addition to this exacerbation of lytic activity, LDV-induced stimulation of NK cells led to an increased IFN-γ production. Accordingly, high plasma IFN-γ levels are observed after infection of both immunocompetent and nude mice, whereas they are reduced by anti-ASGM1 treatment (Markine-Goriaynoff et al., 2002). These characteristics of LDV-induced IFN-γ production correlate with the preventive effect of LDV infection on TEPC.1033.C2 development. To further determine whether this cytokine plays any role in the prevention of plasmacytoma, the effect of LDV infection was therefore analysed in C.129S7(B6)-Ifngtm1Ts/J mice, deficient for IFN-γ on a BALB/c background (Dalton et al., 1993). The kinetics of tumour development were similar in uninfected mice, irrespective of their ability to produce IFN-γ (Fig. 4). In contrast, tumour development was actually enhanced in LDV-infected animals deficient for IFN-γ production, as compared with their LDV-infected WT counterparts (Fig. 4). However, this decrease of virally induced protection was not complete, as TEPC.1033.C2 development was delayed by ~5 days in IFN-γ-deficient mice that had been infected by LDV as compared with uninfected mice (Fig. 4). A similar strong decrease of LDV-induced protection against tumour development in IFN-γ-deficient mice was found in two experiments. These results indicated that IFN-γ was a major, but not exclusive, contributor to the protection conferred by LDV infection.

Surprisingly, in view of the results obtained with nude mice, LDV infection did not protect SCID (severe combined immunodeficient) mice from TEPC.1033.C2 tumours (Fig. 5). This lack of protection was observed in two independent experiments. As the protective effect of LDV against TEPC.1033.C2 was absent in SCID mice, we measured IFN-γ levels that followed infection of these animals. As shown in Fig. 6(a), although SCID mice produced some IFN-γ after LDV infection, the levels were much lower than in BALB/c animals (P=0.0286), which contrasted with normal IFN-γ production of LDV-infected nude mice (Markine-Goriaynoff et al., 2002). Such a reduction of IFN-γ levels was observed in nine independent experiments carried out in mice with a BALB/c background and two experiments in animals with a C57/Bl6 background (not shown). As these results could point towards an involvement of NK/T-cells in cytokine production, IFN-γ levels were also measured after LDV inoculation in the serum of CD1-deficient mice that have no NK/T-cells. Infection was followed by the production of some IFN-γ (Fig. 6b), but the levels remained significantly lower than

**Table 1.** TEPC.1033.C2 growth in the presence of LDV.

<table>
<thead>
<tr>
<th>Culture time (days)*</th>
<th>Cell count (×10⁴)†</th>
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<tbody>
<tr>
<td></td>
<td>Without LDV</td>
</tr>
<tr>
<td>2</td>
<td>1.13±0.06</td>
</tr>
<tr>
<td>3</td>
<td>3.15±0.18</td>
</tr>
<tr>
<td>4</td>
<td>13.5±1.3</td>
</tr>
<tr>
<td>7</td>
<td>83.5±10.9</td>
</tr>
<tr>
<td>9</td>
<td>45.0±3.3</td>
</tr>
</tbody>
</table>

*TEPC.1033.C2 cells (10⁵) were cultured in 2 ml medium with or without 5×10⁶ ID₅₀ LDV. Cells were counted at different times after culture initiation.

†Cells were counted in quadruplicates; results are shown as mean±SEM.

**Fig. 2.** Effect of LDV infection on growth of different cancer cells. (a) Survival of groups of 10 BALB/c mice either uninfected (○) or infected with LDV 1 day before tumour administration (●) was monitored daily after i.p. administration of 10⁵ TEPC.15 cells. (b) Survival of groups of 10 DBA/2 mice either uninfected (○) or infected with LDV 1 day before tumour administration (●) was monitored daily after i.p. administration of 4×10⁵ P815 cells.
in control animals \( (P < 0.03) \). This diminished production of IFN-\( \gamma \) in CD1-deficient mice was found in three out of four experiments. However, due to their different genetic background, tumour progression could not be tested in these animals.

**DISCUSSION**

The link between cancer and viruses is usually recognized in a causal relationship, through transformation of infected cells. However, oncolytic viruses may lead to direct tumour cell death and are indeed considered as a potential therapeutic pathway against cancer (Stanford *et al.*, 2008). Our results indicate that infection with a common non-pathogenic virus like LDV can also enhance cancer immunosurveillance, although this virus cannot infect tumour cells.

Viruses are known as strong activators of the innate immune system. For instance, LDV has already been shown to activate NK cells and to trigger IFN-\( \gamma \) secretion by those cells (Markine-Goriaynoff *et al.*, 2002; Le-Thi-Phuong *et al.*, 2007b). This stimulation of innate responses may result in an enhancement of immunosurveillance, as the effect of LDV on plasmacytoma inhibition was found to be mediated by NK cells, rather than by T-lymphocytes. As the protective effect of the infection was observed only with some tumours, but not for other such as the P815 mastocytoma, it probably depends on mechanisms activated by the virus that efficiently control some, but not all, tumour development. For example, LDV does not protect against P815 mastocytoma that has been reported to be resistant to NK cells (Kiessling *et al.*, 1975). The differences observed between this work and a previous report, where acute LDV infection was found to increase tumour

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**Fig. 3.** Cellular involvement in LDV inhibition of plasmacytoma growth. (a) Survival of groups of 10 BALB/c nu/nu mice either uninfected (○) or infected with LDV 1 day before tumour administration (●) was monitored daily after i.p. administration of 105 TEPC.1033.C2 cells. (b) Survival of groups of 10–11 BALB/c mice either uninfected (○) or infected with LDV 1 day before tumour administration, with (▲) or without (●) anti-ASGM1 treatment, was monitored daily after i.p. administration of 105 TEPC.1033.C2 cells.

**Fig. 4.** Role of IFN-\( \gamma \) in the inhibition of plasmacytoma growth by LDV infection. Survival of groups of six BALB/c (○, ●) and C.129S7(B6)-Igntm1Ts/J (IFN-\( \gamma \)-/-; ▲, △) mice either uninfected (○, ▲) or infected with LDV 1 day before tumour administration (●, △) was monitored daily after i.p. administration of 3×10^4 TEPC.1033.C2 cells.

**Fig. 5.** LDV inhibition of TEPC.1033.C2 growth in SCID mice. Survival of groups of 10 BALB/cBy-SCID mice either uninfected (○) or infected with LDV 1 day before tumour administration (●) was monitored daily after i.p. administration of 105 TEPC.1033.C2 cells.
development (Michaelides & Schlesinger, 1974), might be explained by the distinct route of infection, the microbiological status of the animals resulting in divergent cell activation or the use of a different tumour cell line. However, since no cellular analysis of the LDV effect was performed in the previous report, we can only speculate on the differences in effector cell activation that may have resulted from this distinct experimental protocol. Interestingly, however, in other models, macrophage stimulation as well as cross-reactive antibodies have been suspected to play a similar protective role after LDV infection (Brinton-Darnell & Brand, 1977; Weiland et al., 1990).

As LDV-activated NK cells display a weak ex vivo ability to directly lyse plasmacytoma cells (Markine-Goriaynoff et al., 2002), it was postulated that the secretion of cytokines, especially IFN-γ, was the major mechanism responsible for the in vivo effect of the virus. This hypothesis was supported by the reduced protection that LDV conferred to IFN-γ-deficient animals. Interestingly, both LDV-induced protection against plasmacytoma growth and IFN-γ production were lacking in SCID mice. In addition to T-lymphocytes that cannot develop in SCID animals, and in contrast to NK cells, the NK/T-cell subpopulation is also absent in SCID mice (Miyamoto et al., 2000). Moreover, an involvement of NK/T-cells in LDV-induced IFN-γ production is also supported by the reduced cytokine levels observed in LDV-infected CD1-deficient mice. Therefore, our results may be explained by an involvement of both NK and NK/T-cells in this IFN-γ production. Such a synergy between these cell populations fits well with the report that activation of NK/T-cells by \( x \)-galactosylceramide leads to NK cell stimulation (Eberl & MacDonald, 2000). Although it has been suggested that a cross-talk between NK and/or NK/T-cells and dendritic cells involving IFN-γ may participate in cancer immunosurveillance through enhanced presentation of tumour antigens (Ullrich et al., 2008), such a mechanism is unlikely to explain the protective effect of LDV. Indeed, the persistent protection in nude mice indicates that T-lymphocytes, and therefore antigen presentation, play little if any role in this protection.

It is thus reasonable to consider that, at least under some circumstances, modulation of the microenvironment and especially of the innate immune system by an infection concomitant with the early development of tumour cells may lead to increased cancer immunosurveillance. Thus, in addition to genetic predisposition, environmental factors not related directly to the transformation of tumour cells may determine the outcome of the resulting cancer.

Studies to treat multiple myeloma with expanded NK cells are currently ongoing. As the stimulation of distinct NK cell functions depends on the activation pathway, and especially on the cytokines to which these cells have been exposed (Orange & Biron, 1996), it might be of interest to analyse more closely by which mechanisms LDV infection results in a NK cell stimulation that efficiently prevents plasmacytoma development.

### METHODS

**Animals.** Specific-pathogen-free BALB/c, BALB/c nu/nu, BALB/cBy-SCID (SCID), 129/Sv and DBA/2 female mice were bred at the Ludwig Institute for Cancer Research by Dr G. Warnier and used when 8–12 weeks old. C.129S7(B6)-Ifgmut1Tvj (deficient for IFN-γ on a BALB/c background; Dalton et al., 1993) and C.129S-Cd1tm1Gru/J mice (deficient for NK/T-cells; Smiley et al., 1997) were obtained from the Jackson Laboratories. The project was approved by the local commission for animal care.

**Virus.** Mice were infected by i.p. injection of \( \sim 2 \times 10^7 \) ID\(_{50}\) LDV (Riley strain; ATCC) in 200 µl saline.

**Tumour cells.** Plasmacytoma TEPC.1033.C2 and TEPC.15 cells originally obtained from Dr M. Potter (NIH, Bethesda, MD; Cancro & Potter, 1976) and mastocytoma P815 cells (Dunn & Potter, 1957) were inoculated i.p. injection.

**Antibodies.** Preparation and efficacy of ASGM1 polyclonal antibody have been described previously (Markine-Goriaynoff et al., 2002). NK cell depletion was achieved by injection of 500 µl (\( \sim 8 \) mg) ammonium sulfate-precipitated polyclonal immunoglobulin from immunized rabbit 2 days before tumour cell administration, followed by injection of 300 µl on the same day as tumour cell administration.
IFN-γ assay. IFN-γ sandwich ELISA was performed by using the Mouse IFN-γ DuoSet kit (Genzyme) or CytoSet (Biosource), according to the manufacturer’s instructions.

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