Natural killer cell activation after infection with lactate dehydrogenase-elevating virus

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Early after infection, lactate dehydrogenase-elevating virus (LDV) alters the immune system by polyclonally activating B lymphocytes, which leads to IgG2a-restricted hypergammaglobulinaemia, and by suppressing the secretion of Th2 cytokines. Considering that these alterations may involve cells of the innate immune system and cytokines such as interferon-gamma (IFN-γ), we analysed the effect of LDV on natural killer (NK) cells. Within a few days of infection, a strong and transient NK cell activation, characterized by enhanced IFN-γ message expression and cytolysis, was observed. LDV triggered a large increase in serum IFN-γ levels. Because NK cells and IFN-γ may participate in the defence against virus infection, we analysed their possible role in the control of LDV titres with a new agglutination assay. Our results indicate that neither the activation of NK cells nor the IFN-γ secretion affect the early and rapid virus replication that follows LDV inoculation.

Introduction

In order to control virus infections, infected hosts develop a battery of both adaptive, or specific, and innate immune responses. Depending on the pathogen involved, the control of the infection may rely more on T lymphocyte or natural killer (NK) cell cytolytic responses, on neutralizing antibody production or on the secretion of molecules that interfere with virus replication, such as interferons (IFN). However, some viruses have managed to escape these immune defences and to persist in immunocompetent hosts. A conspicuous example is provided by lactate dehydrogenase-elevating virus (LDV), which persists indefinitely in the circulation of infected mice (Notkins et al., 1966). Interestingly, LDV viraemia peaks at 1 day after infection and then decreases to reach a lower plateau that remains stable for the rest of the life of the infected mouse. It has been proposed that these variations in viraemia depend exclusively on the number of available target cells, since LDV inoculation results in the death of infected cells (Stueckemann et al., 1982). Despite the production of neutralizing antibodies (Notkins et al., 1966) and the induction of effective helper and cytolytic T lymphocytes (Even et al., 1995; van den Broek et al., 1997), these specific anti-LDV responses are not able to control virus replication. As a result, nu/nu mice, or animals with lymphocyte responses suppressed by treatment with irradiation, cyclosporin A or anti-CD4, anti-CD8 or anti-Thy1 antibodies, display similar LDV viraemia as immunocompetent mice (Onyekaba et al., 1989). In contrast, innate cellular responses elicited by LDV infection, such as NK cell activation, have not been as extensively analysed. The stimulation of NK cell activity observed after transfer of tumour cells has been linked to contamination of these cells by LDV (Koi et al., 1981; Leclercq et al., 1987). Moreover, an increased expression of IFN-γ message has been shown in spleen cells obtained several days after LDV infection (Plagemann et al., 1995). However, this IFN-γ expression has been related to T lymphocytes, rather than to NK cells. Since IFN-γ can control the replication of at least some viruses (Smith et al., 1991), the question of a possible role for this cytokine in the control of LDV plasma levels could be asked.
In this report, we examined therefore whether LDV infection actually results in NK cell activation and in IFN-γ secretion by those cells. Our data indicate that the virus indeed triggers an enhancement of cytolyis and IFN-γ expression by NK cells. However, neither the NK cell activation nor the IFN-γ production results in control of virus replication, as found with a new and more accurate procedure for LDV titration.

**Methods**

**Mice.** Specific pathogen-free female CBA/Ht, DBA/2, BALB/c, BALB/c nu/nu and BALB/cBy-SCID (SCID) and isolator-reared 129/Sv mice were raised at the Ludwig Institute for Cancer Research (Brussels, Belgium) and used when 8–13 weeks old. For some experiments, BALB/c mice were purchased from Harlan (Horst, The Netherlands) and NMRI mice were obtained from a local animal facility. IFN-γ receptor-deficient mice (G129), courtesy of F. Brombacher (Max Planck Institute for Immunobiology, Freiburg, Germany), were reared in the same manner as the 129/Sv animals, from which they were initially derived by S. Huang and M. Aguet (Huang et al., 1993).

**Virus.** Mice were infected by intraperitoneal injection of approximately $2 \times 10^7$ ID$_{50}$ of LDV (Riley strain; ATCC) in 500 µl saline.

**Antibodies.** B6507F4 and B6506A7 are IgG2a monoclonal antibodies (mAbs) recognizing the VP3 LDV protein (Coutelier et al., 1986). GK1.5 monoclonal anti-CD4 antibody was made available by F. W. Fitch and obtained through the courtesy of H. R. MacDonald (Dialynas et al., 1983). 53/6.72 anti-CD8 monoclonal antibody was obtained from ATCC (Ledbetter & Herzenberg, 1979). Both anti-CD4 and anti-CD8 antibodies were precipitated with ammonium sulfate. Their in vivo efficiency has been extensively reported (Coulie et al., 1985; Coutelier, 1991; El Azami El Idrissi et al., 1998).

Purified asialoganglioside-GM1 (ASGM1) from bovine brain tissue (Sigma, reference G-3018) was dissolved by sonic dispersion in 0.5 mM NaCl with its amount of methylated bovine serum albumin (Sigma, reference A-1009). For the first immunization, 1.5 mg ASGM1 in complete Freund’s adjuvant was injected intradermally into a rabbit, followed by 0.5 mg ASGM1 in incomplete Freund’s adjuvant for the following immunizations (Kasai et al., 1980). The IgG fraction was obtained from the serum by 50% ammonium sulfate precipitation followed by resuspension in the same volume of PBS, pH 7.2. Different antibody amounts were tested in preliminary experiments of inhibition of IFN-γ secretion. To inhibit NK cell activity, 300 µl antibody was needed and this volume was used in subsequent experiments.

**LDV titration.** LDV titration was performed by a new sensitive particle-counting immuonasay based on the agglutination, by virus particles, of latex beads coated with two different anti-LDV mAbs. This assay greatly reduced the number of mice used for LDV titration; it was easier to perform than conventional methods of injection of sample serial dilutions in groups of mice; and it allowed for more accurate determination of virus titres. It was developed as follows.

**Preparation of latex.** Anti-LDV IgG2a mAbs (B6507F4 and B6506A7) (100 µg) were covalently coupled to 50 µl of carboxylated latex beads of 0.8 µm diameter (Estapor K150, Rhône-Poulenc) by the carbodiimide method (Galanti et al., 1997). The coated particles were stored in aliquots at $-20$ °C. Before use, latex particles were sonicated for 10 s and diluted with glycine-buffered saline–bovine serum albumin (GBS–BSA) buffer (0.1 M glycine, 0.17 M NaCl, 10 mg/ml BSA, 6 mM NaN$_3$, pH 9.2) to adjust the particle number.

**Assay.** Pepsin–HCl reagent [25 µl; 0.3 mol HCl, 5 g porcine pepsin (Sigma) per litre, pH 1.5] was added to 25 µl of serial plasma dilutions and incubated for 10 min at room temperature. To quench the enzyme reaction, 25 µl of Tris (1 M, Merck)–dextran T500 (4%, Pharmacia) reagent was added, together with 25 µl of the reconstituted particle reagent mix containing approximately 30,000 latex bead un-agglutinated monomers. The resulting mixture was incubated at 37 °C for 1 h with continuous vortexing. The reaction was stopped by dilution with 500 µl of GBS. The un-agglutinated particles were counted in an optical counter able to discriminate the un-agglutinated from the agglutinated particles (Masson et al., 1981). The number of un-agglutinated particles was inversely proportional to the LDV concentration. A pool of plasma from NMRI mice infected for 18 h with LDV, with a virus titre of $10^{10.5}$ ID$_{50}$/ml as measured by the classical end-point titration assay, and from control uninfected mice (open symbols), were tested by agglutination assay as described in Methods with latex beads coated with anti-LDV (circles) or anti-CEA (triangles) antibodies. Results are shown as means ± SEM of four measurements.

**Flow cytometry analysis.** After lysis of erythrocytes in 0.83% NH$_4$Cl, spleen and peritoneal cells were incubated for 60 min at 4 °C in 100 µl HAFA buffer (137 mM NaCl, 5 mM KCl, 0.4 mM MgSO$_4$, 0.3 mM MgCl$_2$, 5 mM glucose, 4 mM NaHCO$_3$, 1 mM EDTA, 1 mM phosphate, 20 mM Na$_3$PO$_4$, 100 U/ml penicillin, 100 µg/ml streptomycin, pH 7.4, supplemented with 3% decomplemented FCS) with fluoresceinated DXS antibody (0.2 µg for 10$^6$ cells; Pharmingen). After fixation in 0.62% paraformaldehyde, fluorescence was analysed with a FACScan flow cytometer (Becton Dickinson). Results are shown as percent positive cells after subtraction of spontaneously fluoresceinating cells.

**Cytolytic assay.** $10^5$ 51Cr-labelled YAC-1 and TEPC.1033.C2 cells were incubated with effector cells, at different effector:target ratios in
Results

LDV-induced increase in NK cell population

The effect of LDV on NK cells was first examined by analysing the proportion of spleen and peritoneal cells recognized by DX5 antibody at different times after infection. This antibody has been shown to react with CD49b, a surface antigen expressed on NK cells that are responsible for cytotoxicity (Arase et al., 2001). As shown in Table 1, a significant increase in this cell population was observed in the spleen of 129/Sv mice 4 days after LDV infection. A similar increase was observed in the spleen of BALB/c mice (data not shown). A moderate enhancement of the proportion of CD49b+ cells was found in the peritoneal cells 2 days post-infection (p.i.) (not shown). No modifications were observed at 1 day p.i., either in spleen or in peritoneal cells (not shown).

Enhanced lytic activity of NK cells after LDV infection

To further address the question of NK cell activation after LDV infection, we analysed the lytic activity of spleen and peritoneal cells from LDV-infected mice. Peritoneal cells were collected from control BALB/c mice or from animals infected for 2 days with LDV (○) or from animals infected with LDV (●) were used as effectors in a chromium release assay on labelled YAC-1 or TEPC.1033.C2 target cells.

Table 1. Proportion of NK cells in spleen cells from 129/Sv mice infected with LDV

<table>
<thead>
<tr>
<th>Time after LDV infection (days)</th>
<th>CD49b+ cells (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>9.4 ± 0.5</td>
</tr>
</tbody>
</table>

* Determined by flow cytometry in groups of four 129/Sv mice (mean ± SEM).

Fig. 2. NK-like lytic activity of peritoneal cells after infection with LDV. Pools of peritoneal cells from groups of three control BALB/c mice (○) or from animals infected for 2 days with LDV (●) were used as effectors in a chromium release assay on labelled YAC-1 or TEPC.1033.C2 target cells.
was observed between 1 and 2 days after infection (Fig. 3A). We obtained similar results using effector cells from infected DBA/2 mice (data not shown).

When SCID mice, which do not have cytolytic T lymphocytes, were infected with LDV, the increase in the lytic activity of peritoneal cells against YAC-1 was also observed (Fig. 3B). In addition, the administration of an anti-ASGM1 polyclonal antibody, which eliminates NK cells in vivo, abolished most of the anti-YAC-1 lytic activity from BALB/c or SCID mice infected with LDV (data not shown). We conclude that infection with LDV leads to a large and transient increase in the lytic activity of NK cells.

**LDV-triggered IFN-γ gene expression and IFN-γ production by NK cells**

The effect of LDV on IFN-γ production was determined by measuring this cytokine in the serum of mice at different times after infection. As shown in Fig. 4, a very transient peak of IFN-γ was observed at 18 h p.i. By 38 h p.i., serum IFN-γ had returned to basal levels. An early LDV-induced increase of serum IFN-γ was also observed in CBA/Ht and BALB/c mice (data not shown). The effect of LDV infection on IFN-γ was further analysed at the level of expression with RT–PCR on total RNA extracted from cells obtained at different times post-infection. The gene was found to be expressed shortly after infection in spleen or peritoneal cells of CBA/Ht mice (Fig. 5). Maximal expression was observed 12 h p.i. in peritoneal cells and 6 h later in spleen cells. It was followed by a rapid decrease. Similar results were obtained in 129/Sv mice (not shown).

A similar peak of IFN-γ was observed after LDV infection of nude mice or of animals treated with depleting anti-CD4 or anti-CD8 mAbs 1 day before the infection (Table 3). A large increase in the expression of the IFN-γ gene was also observed in infected nude mice, or in infected animals depleted of their CD4 or CD8 cells, confirming the results obtained with the

![Fig. 3.](image)

**Fig. 3.** (A) Kinetics of LDV-induced lytic activity of spleen and peritoneal cells. The lytic ability of pooled spleen and peritoneal cells from groups of three control BALB/c mice or from animals infected for different times with LDV was determined on YAC-1 target cells. Results are shown for an effector:target ratio of 100. Spleen and peritoneal cells were obtained from separate experiments. (B) Lytic activity of peritoneal cells in LDV-infected SCID mice. The lytic activity of peritoneal cells from groups of three to four control BALB/c or SCID mice or from animals infected with LDV for 1 day was analysed on YAC-1 target cells. Results are shown for an effector:target ratio of 100 (means ± SEM).

![Fig. 4.](image)

**Fig. 4.** Concentration of IFN-γ in the serum of groups (three mice per group) of 129/Sv mice at various times after infection with LDV. IFN-γ levels were measured with ELISA and are expressed as means ± SEM. The dotted line shows the limit of detection.

### Table 2. Effect of NK cell depletion on LDV-induced peritoneal cell lytic activity

<table>
<thead>
<tr>
<th>Expt*</th>
<th>Mouse strain†</th>
<th>LDV‡</th>
<th>Anti-ASGM1§</th>
<th>% Lysis of YAC-1 cells¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>−</td>
<td>−</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>−</td>
<td>60 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>SCID</td>
<td>−</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>−</td>
<td>28 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>1 ± 0.4</td>
</tr>
</tbody>
</table>

* Separate experiments.
† Four mice per group.
‡ Infection on day 0.
§ Treatment with 300 µl anti-ASGM1 polyclonal antibody 1 day before infection.
¶ Determined for peritoneal cells obtained 1 day p.i.; shown for effector:target ratio of 100 (mean ± SEM).
NK cell activation by LDV

Fig. 5. IFN-γ gene expression after LDV infection. Induction of IFN-γ message was analysed with RT–PCR in pools of spleen or peritoneal cells from groups of four CBA/Ht mice.

This indicated that the message expression and production of this cytokine were mostly independent from T lymphocytes. In contrast, the production of IFN-γ was greatly reduced in LDV-infected 129/Sv mice that had been treated by administration of anti-ASGM1 polyclonal antibody (Fig. 6A). The enhancement by the virus of IFN-γ expression was also suppressed by the anti-ASGM1 antibody (Fig. 6B). This treatment had a similar effect in BALB/c animals (data not shown). These results strongly suggest that IFN-γ gene expression and production originate from NK cells of LDV-infected animals.

Effect of NK cells and IFN-γ on LDV viraemia

To analyse the influence of NK cells and IFN-γ on LDV replication, we developed a sensitive assay based on the agglutination by virions of particles coated with anti-LDV monoclonal antibody (Fig. 1). Analysis of the viraemia at 16 and 40 h after LDV inoculation indicated that the kinetics of virus replication were not significantly modified in mice depleted of their NK cells by anti-ASGM1 treatment (Table 4). Similarly, G129 mice developed the same viraemia as their normal counterparts. Together, these results indicate that NK cells and IFN-γ cannot control the early replication of LDV.

Discussion

NK cell activation, leading to both cell-mediated cytoxicity and IFN-γ production, has been reported after infection with several viruses, including lymphocytic choriomeningitis virus (LCMV), mouse hepatitis virus (MHV) and murine cytomegalovirus (MCMV) (Welsh & Zinkernagel, 1977; Bancroft et al., 1981; Natuk & Welsh, 1987; Trinchieri,

Table 3. IFN-γ production and message expression in the absence of T lymphocytes

<table>
<thead>
<tr>
<th>Expt†</th>
<th>Mice‡</th>
<th>Treatment§</th>
<th>Serum IFN-γ (pg/ml)§</th>
<th>Spleen IFN-γ message (arbitrary units)‖</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>—</td>
<td>253 ± 114</td>
<td>5680</td>
</tr>
<tr>
<td>1</td>
<td>BALB/c nu/nu</td>
<td>—</td>
<td>303 ± 227</td>
<td>14943</td>
</tr>
<tr>
<td>2</td>
<td>129/Sv</td>
<td>—</td>
<td>540 ± 371</td>
<td>42504</td>
</tr>
<tr>
<td>2</td>
<td>129/Sv</td>
<td>anti-CD4</td>
<td>933 ± 357</td>
<td>45764</td>
</tr>
<tr>
<td>2</td>
<td>129/Sv</td>
<td>anti-CD8</td>
<td>638 ± 274</td>
<td>16637</td>
</tr>
<tr>
<td>3</td>
<td>129/Sv</td>
<td>—</td>
<td>1638 ± 593</td>
<td>20584</td>
</tr>
</tbody>
</table>

* Separate experiments.
† Four mice per group, infected with LDV on day 0.
‡ 1 mg anti-CD4 or 2.5 mg anti-CD8 monoclonal antibody intraperitoneally, 1 day before infection.
§ Measured by ELISA in sera obtained 15–16 h after infection (mean ± SEM).
‖ Analysed with RT–PCR in pools of spleen cells obtained 15–16 h after infection.
Table 4. Effect of NK cells on LDV replication

<table>
<thead>
<tr>
<th>Expt*</th>
<th>Mouse strain†</th>
<th>Anti-ASGM1 treatment‡</th>
<th>LDV viraemia (agglutinating units)‡‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>16 h p.i.</td>
</tr>
<tr>
<td>1</td>
<td>129/Sv</td>
<td>-</td>
<td>5643 ± 262</td>
</tr>
<tr>
<td>2</td>
<td>129/Sv</td>
<td>+</td>
<td>6639 ± 587</td>
</tr>
<tr>
<td></td>
<td>G129</td>
<td>-</td>
<td>7229 ± 902</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6275 ± 587</td>
</tr>
</tbody>
</table>

* Separate experiments.
† Six mice per group.
‡ 300 µl anti-ASGM1 polyclonal antibody 1 day before infection.
‡‡ Measured by particle counting immunoassay, means ± SEM.

1989; Biron, 1997; Pien & Biron, 2000). It is thus not surprising to observe a similar enhancement of cytolysis after inoculation of LDV, although so far this virus was mainly known to enhance humoral immune responses and to decrease cellular responses (reviewed by Plagemann et al., 1995). This increased cytolytic activity of spleen and peritoneal cells early after LDV infection confirms that similar responses following administration of LDV-containing tumour cell lines could indeed be attributed to the virus (Koi et al., 1981; Leclercq et al., 1987). Moreover, the suppression of this cytolytic activity by treatment with an anti-ASGM1 antibody, and its presence in SCID mice, further demonstrated that NK cells were primarily responsible for this effect. In addition, our observation that the early LDV-induced IFN-γ message and IFN-γ production were not modified in nude mice or in anti-CD4- and anti-CD8-treated animals, but were suppressed by anti-ASGM1 treatment, indicates that this cytokine is also mainly secreted by NK cells.

Our results indicate different kinetics for the activation of diverse NK cell functions elicited by LDV infection, with a very early IFN-γ production, rapidly followed by an increase in cytolytic activity and finally a slightly delayed accumulation of CD49b+ cells. This might be explained by a sequential activation of these functions that may be controlled by distinct cytokines (reviewed by Biron et al., 1999). The enhancement of NK cell-mediated cytolytic activity of spleen and peritoneal cells after LDV infection may be due to secretion of IFN-α/β by infected macrophages (Plagemann et al., 1995; Koi et al., 1981; Leclercq et al., 1987; Heremans et al., 1987). In addition, other cytokines expressed early after LDV inoculation, such as IL-12 (Coutelier et al., 1995), may also be involved in NK cell activation, especially in regard to their cytokine production (Biron et al., 1999). Moreover, IL-15, a growth factor for NK cells secreted by macrophages (Doherty et al., 1996; Biron et al., 1999) that is also produced in response to LDV infection (data not shown), may be responsible for the increase in the proportion of this cell population observed in the spleen of infected animals. Alternatively, we can speculate that distinct cell sub-populations are responsible for the various NK cell functions. For instance, preliminary results obtained in SCID and CD1-deficient mice suggest that the NK T cell subpopulation may be involved in LDV-induced IFN-γ production, in addition to the classical NK cells (data not shown).

NK cells have been shown to play a role in the host defence against viruses, such as MCMV, HSV and influenza virus, but not against LCMV (reviewed by Biron, 1997; Biron et al., 1999). Although it is recognized that LDV viraemia persists despite the development of a T and B cell-mediated anti-viral immune response (Plagemann et al., 1995; Even et al., 1995; van den Broek et al., 1997), little is known about the effects of NK cells on the replication of this virus. Our data, obtained in mice treated with anti-ASGM1 antibody that depletes the NK cell population, indicate that the early and rapid replication of LDV is not controlled by these cells. Moreover, the inability of G129 mice to respond to IFN-γ did not modify virus titres. This result confirms previous observations that, although IFN-γ may protect mice against LDV-induced polioencephalomyelitis (Cafruny et al., 1997), it does not reduce the ability of the virus to replicate in vivo (Cafruny et al., 1999). Together, our results thus indicate that neither NK cells nor the cytokine they produce interfere significantly with the replication of LDV.

Although in most mouse strains infection with LDV does not induce overt pathology, this virus greatly affects the immune system, triggering enhanced humoral responses, with a strong IgG2a-restriction of both anti-viral and non-anti-viral antibodies that are secreted in infected mice (Coutelier & Van Snick, 1985; Coutelier et al., 1986). We recently showed that the production of IgG2a-restricted anti-LDV antibodies, but not of total IgG2a, is controlled by IFN-γ (Markine-Goriaynoff et al., 2000). In addition, LDV modulates the differentiation of T helper lymphocytes by inhibiting Th2 cytokine production (Monteyne et al., 1993, 1997b). LDV also activates macrophages (Meite et al., 2000). LDV-triggered activation of NK cells, and the resulting IFN-γ secretion, may explain some of...
these effects of the virus on B and T lymphocytes. Activated macrophages will then secrete cytokines such as IL-6, IL-12, IL-15 or IL-18 (Coutelier et al., 1995; Markine-Goriaynoff et al., 2001; unpublished data) that may in turn participate in NK cell activation, leading to a reinforcement of the inflammatory characteristics of the immune response developing in the infected animals. Activation of cells in the innate immune system, and especially of NK cells in the early stages after infection may thus play a pivotal role in the immune alterations triggered by this virus.

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