Neonatal infection of mice with lactate dehydrogenase-elevating virus results in suppression of humoral antiviral immune response but does not alter the course of viraemia or the polyclonal activation of B cells and immune complex formation

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Introduction

A unique feature of lactate dehydrogenase-elevating virus (LDV) is its cell specificity for a subpopulation of mouse macrophages (Stueckemann et al., 1992a; Onyekaba et al., 1989b; Plagemann & Moennig, 1992). These macrophages express a surface component that functions as an LDV receptor (Kowalchyk & Plagemann, 1985; Buxton et al., 1988). Lack of the receptor seems to restrict LDV susceptibility to macrophages since cells other than macrophages can be productively infected with isolated LDV RNA (Inada et al., 1993; K.S. Faaberg, C. Even & P. G. W. Plagemann, unpublished). In adult mice, 5 weeks or older, 5 to 10% of peritoneal macrophages are permissive for LDV replication, whereas in mice younger than 2 weeks of age the proportion of permissive peritoneal macrophages is 60 to 80% (Inada & Mims, 1985; Onyekaba et al., 1989b). Infection of these macrophages both in vitro and in vivo is cytocidal (Onyekaba et al., 1989b). Upon infection of a mouse, LDV rapidly replicates in all permissive macrophages resulting in the loss of this population and in plasma LDV titres of up to $10^{10}$ ID$_{50}$/ml 1 day post-infection (p.i.). LDV titres then decrease progressively to about $10^9$ ID$_{50}$/ml during the next 4 to 6 weeks, at which level they remain for the life of the animal (for reviews see Rowson & Mahy, 1985; Plagemann & Moennig, 1992). A persistent infection is maintained by infection of new permissive macrophages that are continuously regenerated at a slow rate from apparently non-permissive precursor cells and escape from all host defence mechanisms, in spite of a vigorous anti-LDV humoral
immune response (Plagemann & Moennig, 1992; Onyekaba et al., 1989a).

In addition to the LDV-specific humoral immune response, LDV infection induces a pronounced chronic polyclonal activation of B cells which results in a permanent elevation in circulating IgG, primarily of IgG2a and IgG2b isotypes (Notkins et al., 1966b; Li et al., 1990; Coutelier et al., 1988; Plagemann & Moennig, 1992). Furthermore, LDV infection results in the formation of immune complexes of low $M_r$ (180K to 300K), which are detected by their ability to bind nonspecifically to ELISA plates in the presence of 0.05% (v/v) Tween 20 (Cafruny et al., 1986a, b; Hu et al., 1992). These small plate-binding immune complexes are distinct from larger infectious anti-LDV antibody–virus complexes which contain practically all virions during the chronic phase of infection (Notkins et al., 1966a; Cafruny & Plagemann, 1982; Hu et al., 1992). The formations of anti-LDV antibodies, of polyclonal IgG2a or IgG2b, and of plate-binding immune complexes occur coincidentally, beginning within a few days p.i. with products attaining maximum serum levels by 4 to 6 weeks p.i. (see Plagemann & Moennig, 1992).

Neonatal infection presents the opportunity to explore several aspects in the interaction of LDV with the host immune system. First, does LDV infection of neonatal mice result in humoral ‘immune tolerance’ to LDV proteins and, if it does, how does the absence of anti-LDV antibodies affect LDV viraemia? The decrease in viraemia after the initial infection has been suggested to reflect, in large part at least, suppression of LDV replication by anti-LDV immune responses (DuBuy et al., 1971; Riley, 1976; Bradley et al., 1991). On the other hand, our evidence has suggested that the level of viraemia during the persistent phase is primarily determined by the rate of generation of new permissive macrophages (Stueckemann et al., 1982b; Hu et al., 1992; Plagemann & Moennig, 1992). Our present results support the latter conclusion; neonatally infected mice fail to generate anti-LDV antibodies, but this has no effect on LDV viraemia.

Second, does infection of neonatal mice result in the polyclonal activation of B cells and, at the same time, in the formation of the small plate-binding immune complexes as observed in mice infected as adults? Our previous indirect evidence suggested that they represent complexes between cellular proteins and autoantibodies formed in the course of the polyclonal activation of B cells rather than between LDV proteins and anti-LDV antibodies (Hu et al., 1992; Plagemann & Moennig, 1992). The latter view is further supported by our present results. The immune complexes are produced concomitantly with polyclonal IgG2a in neonatally infected mice in the absence of anti-LDV antibodies.

### Methods

**Infection of mice with LDV.** Pregnant FVB mice were obtained from the University of Minnesota Transgenic Facility and CSB/M, BALB/c, AKXD-16 and AKXD-20 mice were bred in the University of Minnesota Department of Microbiology animal facility. Litters of FVB, CSB/M and AKXD-16 mice were infected with LDV-P at the time of birth (day 1), at 5 days after birth (day 5), or at 15 days after birth (day 15). An uninfected litter was used as a control. In each group three female pups were retained with the mother. The mother of each infected litter was infected at the same time as the pups and served as a positive adult control. Blood samples were obtained by retro-orbital bleeding and the plasma was stored at $-70^\circ$C. LDV titres were determined using an endpoint dilution assay in mice (Plagemann et al., 1963). Results were reported as ID$_{50}$/ml of plasma. LDV-P was originally isolated in this laboratory (Brinton-Darnell & Plagemann, 1975). Mice were injected intraperitoneally with a dose of about $10^5$ ID$_{50}$ of LDV-P from pools of plasma obtained from groups of 1-day-infected mice.

**Macrophage cultures.** Cultures of peritoneal macrophages were prepared and infected with about 500 ID$_{50}$ of LDV-P/cell as described previously (Stueckemann et al., 1982a).

**Measurement of anti-LDV IgG.** Anti-LDV antibodies were titrated using a fluorescent antibody (FA) staining assay as described by Cafruny et al. (1986a). In brief, peritoneal macrophages from adult mice were cultured on slides and then infected with 100 to 1000 ID$_{50}$ LDV/cell, and at 8 h p.i. were fixed in acetone. Fixed slides were incubated with sequential twofold dilutions of plasma from LDV-infected mice prepared in PBS containing 5% (v/v) fetal bovine serum for 1 h at 37°C, and then were incubated with a 1:75 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) for 1 h at 37°C, and then were viewed using a fluorescence microscope. The anti-LDV FA titre was expressed as the reciprocal of the highest dilution that yielded recognizable staining of 5 to 15% of the total macrophages (the LDV-permissive subpopulation) in LDV-infected cultures. FA titres < 32 were considered to be insignificant.

**Antibody neutralization of LDV infectivity.** Infectivity in mice was then titrated.

**Measurement of total immunoglobulins in plasma and splenocyte cultures and of spleen cell proliferation.** Total immunoglobulins of different isotypes were measured using an ELISA method described previously (Li et al., 1990). ELISA plates (Nunc Immunoplate MaxiSorp F90; Gibco) were coated with goat anti-mouse IgG (Sigma). Plasma was diluted in PBS containing 1% (w/v) BSA (Sigma) and incubated on plates for 2 h at room temperature. The plates were washed and further incubated with alkaline phosphatase-conjugated anti-IgG isotype-specific antibodies (Fisher Scientific). The plates were developed with substrate and $A_{405}$ was measured using an automated plate reader.

For the measurement of the spontaneous proliferation and production of total immunoglobulins in culture, spleens were aseptically removed and minced by passage through a stainless steel wire screen. Red blood cells were removed by lysis with 0.83% (w/v) NH$_4$Cl. The remaining cells were washed twice with RPMI-1640 medium (Gibco) and viable mononuclear cell numbers were determined by counting cells not stained by trypan blue, in a haemocytometer. Spleen cells were cultured in RPMI-10 medium consisting of RPMI-1640 supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (50 μM), l-glutamine (2 mM) and HEPES (10 mM, pH 7.2). Samples of 5 × 10^6 cells in 1.0 ml of RPMI-10 were incubated in 24-well tissue culture plates (Costar) for
5 days and then the culture fluid was analysed for immunoglobulin concentrations. For measuring the spontaneous proliferation of the splenocytes, samples of $1 \times 10^6$ cells in 200 µl of RPMI-10 were cultured in 96-well microtitre plates (Falcon) for 15 h in the presence of 0.5 µCi of [3H]thymidine (dThd; ICN)/well. Then the cells were recovered on glass filters and their radioactivity was counted by liquid scintillation.

**Detection of immune complexes in plasma.** The presence of immune complexes was determined by the non-specific binding to Nunc MaxiSorb ELISA plates as described previously (Cafruny et al., 1986b; Hu et al., 1992). Twofold dilutions of plasma were prepared in PBST (PBS containing 0.05 % v/v Tween 20; Sigma) in wells of the ELISA plates and the plates were incubated overnight at 4 °C. The plates were washed with PBST and incubated for 3 h with alkaline phosphatase-conjugated goat anti-mouse IgG (1/1000 dilution in PBS with 1 % w/v BSA; Sigma). The plates were developed with substrate and the A406 of the fluid was measured using an automated plate reader.

**Preparation and titration of interleukin-3/granulocyte-macrophage colony stimulating factor (IL-3/GM-CSF).** Mouse spleen cell suspensions of $5 \times 10^6$ cells/ml in RPMI-10 were supplemented with 5 to 10 µm pokeweed mitogen (Sigma) and incubated for 1 to 2 days at 37°C. The cell-free culture fluid was assayed for IL-3/GM-CSF by measuring the IL-3/GM-CSF-dependent proliferation of FDCP1 cells (Coligan et al., 1991). Samples of $1 \times 10^6$ FDCP1 cells in 1 ml of RPMI-10 were incubated in 96-well tissue culture plates with various dilutions of the above culture fluid for 24 h. After another 15 h of incubation with 0.5 µCi [3H]dThd/well, the cells were analysed for radioactivity. One unit of IL-3/GM-CSF is defined as the amount that generated 50% of maximum proliferation. The culture fluids from pokeweed mitogen-stimulated spleen cell cultures generally contained about 200 units IL-3/GM-CSF/ml.

**Northern analyses of LDV RNA in tissues.** Spleen, thymus and lymph node cell suspensions were prepared by mincing freshly isolated tissues in RPMI. Mononuclear spleen cells were further enriched by density centrifugation in Ficoll–Hypaque (Sigma). Cell suspensions were washed three times with RPMI. The cells were collected by centrifugation and their RNA was extracted using the acid–guanidinium thiocyanate method as described previously (Chen et al., 1993). Northern analyses were conducted as in earlier studies (Chen et al., 1993). In brief, samples of 10 µg of total glyoxylated RNA were electrophoresed on a 1 % agarose gel, and transferred to a Nytran node cell suspensions were prepared by mincing freshly isolated tissues in RPMI. Mononuclear spleen cells were further enriched by density centrifugation in Ficoll–Hypaque (Sigma). Cell suspensions were washed three times with RPMI. The cells were collected by centrifugation and their RNA was extracted using the acid–guanidinium thiocyanate method as described previously (Chen et al., 1993). Northern analyses were conducted as in earlier studies (Chen et al., 1993). In brief, samples of 10 µg of total glyoxylated RNA were electrophoresed on a 1 % agarose gel, and transferred to a Nytran (Schleicher & Schuell) membrane. For the detection of LDV genomic and subgenomic mRNAs, the membrane was hybridized with a $^{32}$P-labeled cDNA probe of about 1.1 kb, which covers the 3' end of the LDV genome (Chen et al., 1993). For standardization of the hybridization, the blots were stripped and rehybridized with a $^{32}$P-labeled actin probe.

**Results**

The anti-LDV antibody response is suppressed following neonatal infection

FVB mice were infected with LDV within a few hours after birth (day 1) and at 5 days after birth (day 5) and then their anti-LDV immune response was monitored (Fig. 1). In adult mice, anti-LDV antibodies, as measured by the FA staining method, normally appear within the first week p.i. and attain a maximum FA titre of 2000 to 4000 by 4 weeks p.i. and this persists for the lifetime of the mice (Cafruny et al., 1986a; Onyekaba et al., 1989a). FVB mice when infected 5 days after birth generated a normal anti-LDV antibody response; at 4 weeks of age the anti-LDV FA titre of pooled plasma was comparable to that of 4-week-infected adult mice (Fig. 1). In contrast, anti-LDV antibodies were not detected in plasma of FVB mice that were infected within hours after birth, at least not until 7 weeks p.i. (FA titre < 32; Fig. 1). Very low levels of anti-LDV antibodies were detected at 9 weeks p.i. (FA titre = 32), but did not further increase during the 22-week experimental period. Western analyses have shown that plasma from chronically infected mice contains primarily antibodies to the envelope glycoprotein(s) and nucleocapsid protein of LDV. Monoclonal antibody studies have shown that antibodies to these structural proteins are detected by the FA staining method (Cafruny et al., 1986a; Harty & Plagemann, 1988; Coutelier et al., 1986). Thus, the FVB mice failed to generate antibodies to these LDV proteins. Furthermore, at 22 weeks p.i., the plasma of neonatally infected mice did not contain detectable levels of antibodies that neutralize LDV infectivity, whereas the plasma of the companion mice infected at 5 days p.i. neutralized LDV infectivity by about 99 %. Comparable results were obtained with C58/M and AKXD-16 mice; significant levels of anti-LDV antibodies were not detected in the plasma of mice infected immediately after birth until at least several weeks p.i. and thereafter only at a hardly detectable level (data not shown).

**Lack of formation of anti-LDV antibodies in neonatally infected mice is not associated with increased LDV replication**

The failure of neonatally infected FVB mice (day 1 group) to mount a humoral immune response to LDV presented the opportunity for further examination of the effect of a lack of anti-LDV immune responses on LDV replication, as measured by the level of viraemia in persistently infected mice. The steady-state level of viraemia in these mice represents an equilibrium between the rate of production of new infectious virions and the rate of removal of infectious virus from the circulation either by immune or non-specific mechanisms. Suppression of LDV replication by humoral or cellular anti-LDV immune responses would be expected to lower viraemia, whereas increased production of LDV by infected permissive macrophages would be expected to result in increased viraemia. The results in Fig. 1 show that the plasma titres of LDV in individual mice were comparable whether the FVB mice were infected at day 1, 5 or 15 after birth, that is whether or not they mounted an anti-LDV immune response. This finding further supports our conclusion that the anti-LDV immune response does not significantly affect LDV replication in
vivo (Plagemann & Moennig, 1992). In contrast, as documented by the following sections, the level of viraemia is determined by the availability of permissive macrophages.

**Relationship between the availability of LDV-permissive macrophages in the peritoneal cavity and the level of viraemia**

Although the plasma LDV titres of FVB mice infected at 1, 5 or 15 days after birth did not differ significantly from each other, they were on the average about 10-fold higher until about 6 weeks of age than those of their mothers infected at the same time. Thereafter, the LDV titres of offspring and mothers became indistinguishable (Fig. 1). The plasma LDV titres of the mothers were about $10^9$ ID$_{50}$/ml at 1 day p.i. and then decreased in the next 2 to 4 weeks to a lower persistent level, as is typical for LDV infections of all mouse strains (Plagemann & Moennig, 1992). Viraemia of the offspring could only be determined, without sacrificing the mice, from 2 to 3 weeks of age. The higher viraemia in the offspring was probably due to a greater availability of permissive macrophages since the time period of higher viraemia was related to the age of the mice rather than the time p.i. and coincided with the time period when the proportion of LDV-permissive macrophages in the peritoneum was high (60 to 80%) and then decreases progressively to 5 to 10% (Inada & Mims, 1985; Onyekaba et al., 1989b).

The notion that the level of viraemia during persistence is primarily determined by the availability of permissive macrophages is further supported by the following findings. Treatment of LDV-infected mice with either cyclophosphamide or dexamethasone has been previously shown to result in an approximately 10-fold increase in plasma LDV for as long as the mice are treated (Dubuy et al., 1971; Onyekaba et al., 1989a). In the present study, we show that treatment of uninfected BALB/c mice with either drug for 2 days or longer resulted in a marked increase in the proportion of LDV-permissive macrophages in the peritoneum (Table 1, Fig. 2) without significantly affecting the total number of macrophages harvested from the mice. Similarly, repeated injection of mice with IL-3/GM-CSF, which has been shown to cause an up to 15-fold increase in the number of macrophages harvested from the mice. Thrice-daily intraperitoneal injection of two 4-week LDV-infected AKXD-20 mice with 200 units of IL-3/GM-CSF for 7 days increased their plasma LDV titre from $10^5$ and $10^6$ ID$_{50}$/ml to $10^7$ and $10^8$ ID$_{50}$/ml of plasma, respectively. This treatment had no effect on the anti-LDV antibody titre of the plasma (FA titre = 2048 to 4096). This result suggests that the increases in LDV
Table 1. Effects of treatment of mice with cyclophosphamide and dexamethasone on the LDV permissiveness of their peritoneal macrophages*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDV-infected macrophages (% of total ± s.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>Cyclophosphamide, once</td>
<td>18.8 ± 0.9</td>
</tr>
<tr>
<td>Cyclophosphamide, twice</td>
<td>13.2 ± 0.8</td>
</tr>
<tr>
<td>Dexamethasone, once</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>Dexamethasone, twice</td>
<td>9.7 ± 0.3</td>
</tr>
</tbody>
</table>

*Groups of two 2-month-old BALB/c mice were injected intraperitoneally with 200 mg cyclophosphamide/kg or 100 mg dexamethasone/kg once or twice in a weekly interval or remained untreated. Two days after the last injection, the peritoneal exudate cells were harvested and cultured on triplicate coverslips. The next day the cultures were infected with 500 ID₅₀ of LDV-P/cell and at 8 h p.i. examined by FA staining for the proportion of LDV-infected cells (see Fig. 2). A total of 500 cells were counted per coverslip and the values represent means of the triplicate cultures ± s.E.M.

Viraemia resulted from an increased generation of permissive macrophages stimulated by the cytokine treatment.

It was also apparent from the data in Fig. 1 that the persistent viraemia in the chronically infected FVB mice of about 10⁷ ID₅₀/ml was significantly higher than the viraemia previously observed in chronically infected BALB/c, Swiss and C58/M mice (10⁴ to 10⁵ ID₅₀/ml; Cafruny et al., 1986a; Harty & Plagemann, 1988; Onyekaba et al., 1989a) and in other diverse mouse strains (Rowson & Mahy, 1985). This difference was unrelated to the humoral antibody responses of the mice since the neonatally infected mice lacked anti-LDV antibodies, whereas the plasma anti-LDV FA titres of the mice infected at 5 days after birth were about the same as those of their mothers and those generated in other mouse strains (Cafruny et al., 1986a; Onyekaba et al., 1989a). It seems more likely that the higher viraemia in FVB mice was due to a more efficient regeneration of LDV-permissive macrophages in these mice than in other mouse strains but we have not been able to prove this point by analysing the LDV permissiveness of peritoneal macrophages of the mice. The numbers of peritoneal exudate cells harvested per adult mouse (4 x 10⁶ to 6 x 10⁶) were similar for FVB mice as for other mouse strains of similar age as was the proportion of LDV-permissive cells in the total adhering population (5 to 10%). The most likely explanation for this finding is that during the persistent infection new permissive macrophages become generated mainly in lymphoidal tissues rather than the peritoneum (see below and R. R. R. Rowland, G. Anderson & P. G. W. Plagemann, unpublished).

Virus replication in the thymus

The induction and maintenance of neonatal immunological tolerance generally assumes that antigen is continuously present in the primary immune organs, such as the thymus (for T cell tolerance) and bone marrow (for B cell tolerance). FA staining of frozen tissue sections has shown that at 12 to 24 h p.i. LDV replication occurs in all tissues of a mouse, including the...
thymus, but that practically all infected cells disappear by 48 h p.i. (Porter & Porter, 1971; Inada & Mims, 1985; Chan et al., 1989), these findings being consistent with the cytocidal nature of LDV replication in macrophages (Plagemann & Moennig, 1992). Similarly, LDV-infected cells have been detected in sections of the spleen by in situ hybridization and by electron microscopy at 1 day p.i., but not at later times (Stueckemann et al., 1982a; Chan et al., 1989) and the same applies to the detection of LDV genomic and subgenomic mRNAs in the spleens by using Northern hybridization analysis (Contag & Plagemann, 1989; Kuo et al., 1992). Thus, little is known about the site of LDV replication during chronic infection. LDV replication in the persistently infected adult BALB/c, Swiss and C58/M mice used in these studies seems too limited, probably involving only between 100 and 1000 newly generated permissive macrophages per day (Stueckemann et al., 1982b) to be detectable by these methods. The relatively high LDV titres in the persistently infected young FVB mice (Fig. 1) suggested that it might be possible to detect LDV replication in tissues of these mice during the persistent phase of infection.

FVB mice were infected with LDV at birth and sacrificed at 1 and 14 days p.i., RNA was extracted from spleen, thymus and brain and analysed by Northern hybridization using a cDNA probe representing the 3' end of the viral genome. This probe hybridizes to genomic LDV RNA as well as to all subgenomic mRNAs (Chen et al., 1993) which ensures that LDV RNA replication in tissues is detected rather than RNA from blood-borne virus. The results in Fig. 3(a) show that genomic LDV RNA (approximately 14 kb) as well as all seven subgenomic mRNAs were readily detected in spleen and thymus, but also were detectable at low levels in the brains of these mice at 1 day p.i. They were also present in these tissues at 2 weeks p.i., but only at barely detectable levels. For comparison, a Northern blot of RNA extracted from macrophages infected with LDV for 8 h is shown in Fig. 3(b). The heavy bands of about 4.4 kb in Fig. 3(a) represent non-specific hybridization of the probe to 29S rRNA which we generally observe with total RNA extracted from tissues. The continued presence of LDV mRNAs in thymus and other tissues suggests that LDV antigens are generated continuously in these tissues throughout the persistent infection.

**B cells become polyclonally activated in neonatally infected mice**

The polyclonal activation of B cells during LDV infection of adult mice results in significant permanent increases in IgG, predominantly IgG2a and/or IgG2b, up to 5 to 10 mg/ml (Li et al., 1990; Coutelier et al., 1988, 1990). The results in Fig. 4 demonstrate that elevated plasma IgG2a and IgG2b levels were observed in neonatally infected FVB mice (day 1) and that these were higher than the levels observed in companion uninfected mice when analysed at 5 weeks of age. The difference in plasma IgG levels between uninfected mice and LDV-infected mice was detected only when the mice were maintained under pathogen-free conditions. In another experiment, in which the mice were maintained under normal housekeeping conditions, the levels of plasma IgG2a plus IgG2b had increased to 2 to 3 mg/ml at 5 weeks after birth whether the mice were uninfected or LDV-infected at 1, 5 or 15 days of age. We attribute this increased production of IgG2 to random exposure to other agents in the environment which obscured the polyclonal B cell activation induced by LDV.

Other indicators of polyclonal activation of B cells in adult mice after LDV infection are increases in B cell proliferation and production of IgG2a and IgG2b in splenocyte cultures (Coutelier et al., 1990; Li et al., 1990). Both of these changes begin to become detectable within a week p.i. The results in Table 2 show that spleen
Infected at age

Uninfected
Day 1
Day 5

Uninfected
Day 1
Day 5

Uninfected
Day 1
Day 5

IgG2a
IgG2b
IgG2a + IgG2b

Plasma IgG2 (µg/ml)

Fig. 4. Total circulating IgG2a and IgG2b in FVB mice infected in groups of three with LDV at 1 and 5 days after birth and in three companion uninfected mice. The animals were maintained under relatively pathogen-free conditions in which all cages, food, bedding, water and cage covers were sterilized prior to use and the mice were kept in an area isolated from other mice. Plasma was obtained from these mice at 5 weeks after birth and analysed for concentrations of IgG2a and IgG2b. The values are means of the results from three mice.

The results indicate that the polyclonal activation of B cells is induced by LDV infection in the absence of an anti-LDV antibody response.

**Formation of ELISA plate-binding immune complexes correlates with formation of polyclonal IgG2a/IgG2b and occurs in the absence of anti-LDV antibodies**

The formation of low Mr, ELISA plate-binding immune complexes in LDV-infected mice, like the polyclonal activation of B cells, is a T cell-dependent process (Hu et al., 1992). The results in Fig. 5(a) show that similar levels of plate-binding immune complexes developed in neonatally infected FVB mice as found in mice infected at 5 or 15 days of age or as adults, even though the neonatally infected mice did not mount a humoral anti-LDV immune response (Fig. 1). Thus the formation of these immune complexes occurs independently of the anti-LDV immune response and the complexes do not contain anti-LDV antibodies. The data in Fig. 5(a) also document that such immune complexes did not develop in uninfected FVB mice. The isotype specificity of the IgG present in the immune complexes formed in the infected mice is shown in Fig. 5(b and c). The complexes contained primarily IgG2a and this is observed in other mouse strains (Hu et al., 1992), except for the neonatally infected mice which possessed mainly IgG2b-containing immune complexes. This difference may not be related to the neonatal infection since similar differences have been observed in groups of adult Swiss mice (Hu et al., 1992). Other experiments have shown that ELISA plate-binding immune complexes also developed in neonatally infected

Table 2. Comparison of spontaneous proliferation and IgG production by cultures of spleen cells from FVB mice infected at various ages and uninfected mice*

<table>
<thead>
<tr>
<th>Infected at age (days)</th>
<th>[³H]dThd incorporation (c.p.m.)</th>
<th>IgG produced (ng/ml)</th>
<th>7-day LDV-infected mice</th>
<th>IgG produced (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG2a</td>
<td>IgG2b</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30900</td>
<td>1.6</td>
<td>0.9</td>
<td>92300</td>
</tr>
<tr>
<td>5</td>
<td>ND†</td>
<td>3.5</td>
<td>0.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

*FVB mice maintained under pathogen-free conditions were infected with LDV at the indicated ages. Spleens were removed 7 days p.i. from five and three mice infected at 1 day and 5 days of age, respectively, and from uninfected companion mice. Spleen cell suspensions were prepared from the pooled spleens and assayed for spontaneous proliferation ([³H]dThd incorporation). The cells were also cultured for 5 days (without concanavalin A) and the culture fluid was assayed for IgG2a and IgG2b. All values are means of results from triplicate cultures.

†ND, Not determined.
Fig. 5. Circulating plate-binding immune complexes containing total IgG (a), IgG2a (b) or IgG2b (c) in FVB mice infected with LDV at 1 (●), 5 (▲) and 15 (▼) days after birth, in uninfected companion mice (△, uninfected adult; ▲, uninfected neonate) and an LDV-infected adult mouse (○). The experiment is described in the legend to Fig. 1. Immune complexes were measured by the non-specific binding of IgG to ELISA plates in the presence of 0.05% (v/v) Tween 20. Bound total IgG, IgG2a and IgG2b were detected by reaction with alkaline phosphatase-conjugated goat anti-mouse IgG-, IgG2a- and IgG2b-specific antibodies, respectively. All A405 values are means of three analyses for plasma pooled from three mice or a single adult mouse.

C58/M and AKXD-16 mice that failed to generate anti-LDV antibodies (data not shown).

Discussion

Our results demonstrate that mice neonatally infected with LDV fail to generate a significant anti-LDV antibody response (Fig. 1). A similar unresponsiveness has been observed in mice congenitally or neonatally infected with other viruses, such as murine leukaemia virus (MuLV; Zijstra & Melief, 1986; Korostoff et al., 1990) or lymphocytic choriomeningitis virus (LCMV; Buchmeier et al., 1980; King et al., 1992). Persistent immune tolerance requires the continuous elimination or inactivation of LDV-specific B cell clones and/or the elimination of LDV-specific CD4+ T-helper cells in the thymus (Nossal, 1989). In this context, LDV antigens would inactivate B and T cells by binding lymphocyte antigen receptors during a susceptible stage of development (Nossal, 1989). To ensure continuous removal of newly arising T and B cells, and thereby to maintain tolerance, would require the continuous presence of antigen in the thymus (King et al., 1992). In the case of viruses, a most efficient source of viral antigen occurs via virus replication in the thymus, and the replication of both MuLV (Korostoff et al., 1990) and LCMV (King et al., 1992) in lymphoid cells in the thymus has been demonstrated. The target of LDV replication is the macrophage, a widely distributed cell population capable of inducing T cell tolerance in the thymus (Jayaraman et al., 1992). LDV replicates in macrophages in the thymus and bone marrow during the first day after infection of a mouse and our present results suggest that LDV replication occurs in the thymus during the persistent phase which would provide a continuous supply of LDV antigens. It is not known whether LDV-specific cytotoxic T lymphocytes, if they are generated in LDV-infected mice (Plagemann & Moennig, 1992), are suppressed in neonatally infected mice. However, such an effect could be anticipated since LDV antigenic peptides would be expected to be continuously presented in the context of a major histocompatibility complex class I antigen by LDV-infected macrophages in the thymus.

The lack of formation of anti-LDV immune responses in neonatally infected mice in the absence of general T cell defects allowed us to obtain additional proof for our conclusion that LDV replication during the chronic phase is determined by the rate of regeneration of permissive macrophages and is not restricted by the anti-LDV immune response. This conclusion was previously indicated by our finding that the time course and level of LDV viraemia in infected immunodeficient nude mice and in mice in which anti-LDV immune responses were suppressed by depletion of the mice of CD4+ T helper cells was the same as in companion immunocompetent mice (Onyeakaba et al., 1989a). The present results show that the level of LDV viraemia is also normal in neonatally infected mice that are immune-tolerant to LDV proteins (Fig. 1). Bradley et al. (1991) observed that plasma LDV titres were 10- to 100-fold higher in SCID mice than in wild-type mice and concluded that the higher LDV titres were due to the lack of formation
of anti-LDV antibodies which normally suppress LDV replication in SCID mice. These investigators, however, also reported that a relatively high proportion of peritoneal macrophages in these SCID mice were LDV-permissive (28%). It seems possible therefore that the lack of both T and B cells in SCID mice leads to an increase in the generation of macrophages and that the higher viraemia in these mice arises from the availability of a greater number of LDV-permissive macrophages than is found in wild-type mice. An increased generation of LDV-permissive macrophages probably also accounts for higher viraemia in cyclophosphamide- and dexamethasone-treated mice than in untreated mice (Table 1) and for the higher viraemia in FVB mice than in other mouse strains. These conclusions are further supported by the finding that plasma LDV titres are also higher in infected mice during the first several weeks of age, a period which coincides with a high proportion of LDV-permissive macrophages being present in the peritoneum and with the time period when peritoneal macrophages exhibit self-sustained growth (Metcalf et al., 1992). Furthermore, repeated injection of chronically infected mice with IL-3/GM-CSF, which is known to stimulate macrophage generation (Metcalf et al., 1987, 1992), raised their plasma LDV levels more than 10-fold.

The means by which LDV replication in macrophages escapes all host defence mechanisms is not clear but is of considerable interest with respect to persistent virus infections. Antibodies that neutralize LDV infectivity in vitro appear in infected mice about 1 month p.i., but neutralization is very inefficient and requires binding of many antibody molecules per virion (Plagemann et al., 1992). Passive transfer experiments have shown that these antibodies do not protect mice from LDV infection or significantly affect LDV replication in macrophages in vivo (Onyekaba et al., 1989a; Harty & Plagemann, 1988; Plagemann & Moennig, 1992). However, anti-LDV antibodies to the envelope glycoprotein do bind to virions so that during chronic infection all infectious virions in the circulation are associated with antibodies (Notkins et al., 1966a; Cafruny & Plagemann, 1982; Hu et al., 1992). There is evidence that neutralization-escape variants may become selected during LDV persistence (P. Monteyne & J.-P. Coutelier, personal communication). However, even if they arise, their replication would be limited by the availability of permissive macrophages.

The virion–antibody complexes clearly differ from the 180K to 300K ELISA plate-binding immune complexes formed in LDV-infected mice, both in size and composition (Hu et al., 1992). The finding that production of both polyclonal IgG2a or IgG2b and of ELISA plate-binding immune complexes occurs to about the same extent in neonatally infected mice in the absence of anti-LDV antibodies as occurs in non-immunotolerant mice infected when 5 days of age or older indicates that the polyclonal activation of B cells is independent of the antiviral humoral immune response. Thus the polyclonal activation of B cells is not simply a ‘bystander’ effect (Ahmed & Oldstone, 1984) mediated by LDV-specific T helper cells. The finding also supplies unequivocal proof that the plate-binding immune complexes do not contain anti-LDV antibodies. Furthermore, as in previous experiments (Hu et al., 1992), the isotype specificity of the IgG that is present in the immune complexes reflected that of the IgG produced by the polyclonally activated B cells. However, only a small proportion of the polyclonal IgG2a or IgG2b that is produced is present in these immune complexes (Hu et al., 1992). These findings support our view that the immune complexes consist of autoantibodies formed in the course of the polyclonal activation of B cells and their cellular antigens. That various types of autoantibodies are produced during a persistent LDV infection has been demonstrated (Grossman et al., 1989; Weiland et al., 1987; Cafruny & Hovinen, 1988). Whether these immune complexes exert any pathogenic effect is not known. They seem too small to be trapped in the kidney and since they contain mainly IgG2a they do not efficiently fix complement and their presence does not lead to immune complex disease (Rowson & Mahy, 1985). However, primary humoral antibody responses are impaired in chronically infected mice (Michaeldes & Simms, 1980; Riley et al., 1976) and the immune complexes could play a role in this effect. Similar plate-binding immune complexes are formed in mice infected with MAIDS retrovirus (Even et al., 1992), are present in antinuclear antibody-positive human sera (Cafruny et al., 1986b) and could be formed in other persistent infections in association with polyclonal activation of B cells and in other autoimmune disorders.

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