Lactate dehydrogenase-elevating virus: an ideal persistent virus?

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Introduction

Lactate dehydrogenase-elevating virus (LDV) seems an "ideal" persistent virus in the sense that it invariably establishes a life-long, viremic infection in mice regardless of strain, age or sex, escaping all host defense mechanisms and without causing significant harm to the host (for other reviews see [7, 58, 59, 67, 68]). A productive, cytocidal replication of LDV is restricted to a subpopulation of resident macrophages present in practically all tissues. Permissive macrophages are continuously renewed in lymphoidal tissues, the liver and testis, and support the persistent infection. This subpopulation of macrophages seems nonessential for the host. It functions in clearing lactate dehydrogenase (LDH) from the circulation. Continuous destruction of this subpopulation results in a five- to tenfold elevation of plasma LDH levels, hence the name LDV [59, 63]. LDV establishes a persistent infection in spite of being a highly cytocidal virus. Each infected macrophage releases about 10^4 infectious units within 12–18 h post infection (p.i.). High titers of anti-LDV antibodies are generated within 2 weeks p.i., but neutralizing antibodies appear only at about 1 month p.i. and their neutralization of viral infectivity can only be demonstrated in vitro and is very inefficient. Anti-LDV cytotoxic $CD8^+$ T cells are also generated rapidly, but have disappeared by 30 days p.i. and cannot be restimulated in persistently infected mice. Throughout the infection, large amounts of virus accumulate in newly formed germinal centers of the spleen and lymph nodes coincidental with a polyclonal activation of B cells that results in a permanent elevation of plasma IgG2a and the formation of autoantibodies and of low molecular weight immune complexes. During the persistent infection, the ability of the mice to mount primary and secondary antigen-specific humoral immune responses is slightly reduced. The only factor limiting the success of LDV as a virus is the relative inefficiency of its transmission between mice.

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Physical, chemical and molecular properties

LDV belongs to a new, as-yet-unnamed family of positive-stranded RNA viruses presently classified as the genus Arterivirus [58, 59]. Other members of this family are equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV) and simian hemorrhagic fever virus (SHFV). Members of this group are enveloped viruses which possess a diameter of 50–65 nm, a relatively smooth surface and a spherical nucleocapsid core with a diameter of 25–35 nm ([8, 34, 59, 68] see also Fig. 2B). The nucleocapsid is composed of a 13- to 15-kb, linear, single-stranded, polyadenylated RNA of positive polarity and a 12- to 14-kDa nucleocapsid protein (N or VP-1). The envelope contains a nonglycosylated 18- to 20-kDa protein (M or VP-2), one primary glycoprotein (VP-3P) of 25–60 kDa and one minor glycoprotein (VP-3M) of similar size (see Fig. 1B and later).

The general organization and expression of the genomes of this group of viruses (e.g., Fig. 1A for LDV) resemble those of the coronaviruses. Genome expression involves the formation of a 3′ coterminal nested set of six or seven subgenomic mRNAs; mRNAs 2–7 for all four viruses (see Fig. 1A) and one additional mRNA (1-1) for LDV [14, 43]. Each subgenomic mRNA carries a noncoding 5′ leader of 156–212 nucleotides (nts) derived from the 5′ end of the viral genome and a noncoding segment at the 3′ end which is polyadenylated (Fig. 1A) [15]. The 5′ leader becomes joined to the body of each subgenomic mRNA within or just upstream of a single specific, short nucleotide sequence (a heptanucleotide in the case of LDV) at its 3′ end. It is joined to the bodies of the subgenomic mRNAs at a completely or partially homologous segment preceding each of the open reading frames (ORFs) 1-1 and 2 to 7 [14, 20, 48]. However, the mechanism of formation of the subgenomic mRNAs has not been elucidated (see [13, 58, 59]).
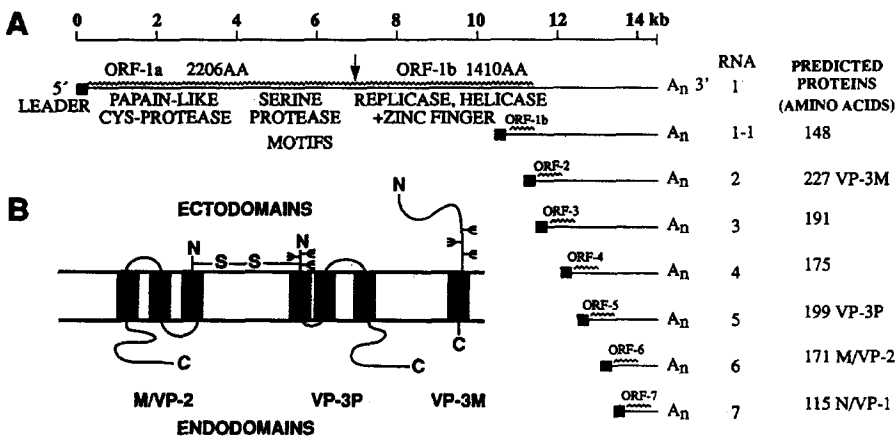

Fig. 1. Organization of the lactate dehydrogenase (LDH)-elevating virus (LDV)-P genome (A) and of the predicted structural proteins in the virion envelope (B). In A, the *boxes* indicate the 5′ leader and A_n the 3′ poly(A) tail on each mRNA. The number of amino acids of the products predicted by the open reading frames (ORFs) and their presumed functions or functional motifs are indicated. The *arrow* indicates the location of the ORF 1a termination codon and of the slippery sequence. In B, the *black boxes* indicate transmembrane segments, *S-S* represents the disulfide bond between the ectodomains of VP-3P and M/VP-2, ψ represents the oligosaccharide side chains, and *N* and *C*, the N-terminal and C-terminal ends of the proteins, respectively

Each subgenomic mRNA most likely expresses a single protein encoded by the most 5' ORF (Fig. 1A). Each of ORFs 2 to 7 overlaps to a various extent with its neighbors (up to 217 nts) and the ORFs are read in alternate frames. Sequence analyses of the genomes of two strains of LDV, LDV-C [30] and LDV-P [57], and single strains of EAV [20] and PRRSV [49] have allowed identification of the coding sequences of the nonstructural and structural proteins of these viruses (e.g., for LDV-P, Fig. 1A) and conclusions as to the conformation of the envelope proteins in the virion envelope (Fig. 1B and [27, 28]).

The nucleocapsid protein (N/VP-1) of all four viruses is encoded by the most 3' ORF (ORF-7; see Fig. 1A). The N proteins are phosphoproteins of similar size (110–128 amino acids; see later Table 1) and are highly basic (pI 10.4–12.3) which probably facilitates their interaction with genomic RNA in the assembly of the nucleocapsids. ORF 6 of these viruses encodes the nonglycosylated envelope proteins M/VP-2 (162–173 amino acids; see Fig. 1A). The M proteins are also unusually basic (pI 10.2–11.9). Their structures resemble those of the coronavirus M proteins in that they possess three closely adjacent transmembrane segments near the N-terminal end, the most N-terminal of which probably functions as an uncleaved signal sequence (Fig. 1B). The postulated N-terminal ectodomains of the M proteins of these viruses are very short (only 9–13 amino acids).

The ORF 5 protein has been shown to be the primary envelope glycoprotein of EAV (G_L; L = large) and the ORF 2 protein is a second minor envelope glycoprotein ($G_{:S}$; S = small) [22]. The same applies to LDV (the corresponding LDV proteins are tentatively designated VP-3P and VP-3M, respectively, because they are of about the same size [27] (Fig. 1A). The ORF 5 proteins of these viruses exhibit similar structures which are unique for a major viral envelope glycoprotein [27, 28]. They are predicted to traverse the lipid bilayer three times, just as M/VP-2, also exposing only relatively short ectodomains (see Fig. 1B). In the case of LDV and PRRSV, a segment of only about 30 amino acids of VP-3P with one to three potential N-glycosylation sites would project externally after cleavage of a signal peptide (Fig. 1B). This might explain the smooth appearance of the outer surface of the virions as seen in electron micrographs [8, 34]. The ectodomain of G_L of EAV is somewhat larger (about 90 amino acids) [22]. Unusually large oligosaccharide side chains are linked to the N-glycosylation sites in the ectodomains of VP-3P of LDV [27] and G_L of EAV [22]. These have been identified as polylactosaminoglycans [22] similar to those on the NB protein of influenza B virus [76]. The presence of these large heterogenous polylactosaminoglycans explains the heterogenous size of the VP-3P/G_L proteins (25–42 kDa) [8, 12, 22]. The ORF 2 proteins are more conventional envelope glycoproteins. They possess a signal peptide and a C-terminal transmembrane segment with one to four potential N-glycosylation sites in between (Fig. 1B).

Polyclonal and monoclonal antibodies that neutralize LDV and EAV in vitro are all directed towards VP-3P and G_L, respectively [6, 12, 21, 27, 31], probably to epitopes located in the ectodomains of these glycoproteins (see Fig. 1B). VP-3P in LDV virions [28] and G_L in EAV virions (de Vries, personal communication) are linked by disulfide bonds to M/VP-2, probably involving single Cys residues in the ectodomains of the two proteins which are conserved in the viruses of this group [28] (see Fig. 1B). Breakage of the disulfide bonds results in complete inactivation of LDV infectivity [28]. These results combined with the specificity of the neutralizing antibodies suggest that the disulfide-linked ectodomains of VP-3P and M/VP-2 form a site for the attachment of LDV to a cell surface receptor. In contrast, the relatively

large endodomains of M/VP-2 and VP-3P/G_L (Fig. 1B, 73–82 amino acids) may play a role in directing the budding of the nucleocapsids into the internal space of cytoplasmic membrane vesicles (see Fig. 2B). The structures of the two major envelope proteins of this group of viruses and their linkage by disulfide bonds (Fig. 1B) is unique among viruses. The function(s) of the nonstructural ORF 3 and ORF 4 glycoproteins are unknown. The largest subgenomic mRNA (1-1; Fig. 1A) is unique for LDV. It carries the 3′ end of ORF 1b and potentially encodes a 148-amino acid protein [14] but whether this protein is expressed in infected cells is not clear.

The 5′ ends of the genomes of the viruses of this group ($\sim$ 10–11 kb, see Fig. 1 A) encode two large proteins, 1a (1727–2396 amino acids) and 1b (1410–1463 amino acids). The ORF 1b protein is expressed via a frameshift mechanism [20]. The ORF 1a/1b proteins possess several functional motifs (Fig. 1A); papain-like cysteine proteinase (PCP) and 3C-like serine proteinase motifs in the ORF 1a protein and replicase, helicase and zinc finger motifs in the ORF 1b protein. They are cleaved into functional units by the virion proteinases. The N-terminal end of the EAV ORF 1a protein possesses one PCP which autocatalytically releases the N-terminal 29-kDa end of the protein (nsp1) [69]. The ORF 1a proteins of LDV and PRRSV possess two PCPs which release N-terminal products of about 21 (nsp1α) and 26 kDa (nsp1β) [19]. The serine proteinase is more likely to function in *trans* and seems responsible for further processing of the ORF 1a protein and of the ORF 1b protein [70].
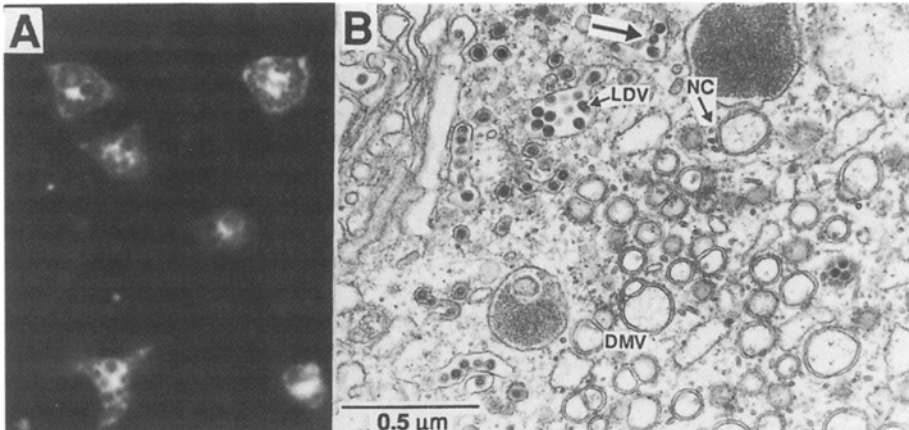

Fig. 2. Indirect fluorescent antibody staining of a 7-h LDV-infected culture of peritoneal mouse macrophages (A) and electron micrograph of a section of a 20-h-infected macrophage (B). In A, the culture was fixed in acetone and sequentially incubated with plasma from 2-month LDV-infected mice and fluorescein isothiocyanate-conjugated anti-mouse IgG [12]. The electron micrograph in B shows a perinuclear area with double-membrane vesicles (*DMV*), free nucleocapsids (*NC*), nucleocapsids budding into a single-membrane vesicle (*arrow*) and Golgi membranes (*upper left side*)

LDV replication and persistent infection of mice

Infections of primary cell cultures showed that only subpopulations of macrophages from the peritoneum and various tissues of mice support LDV replication to a significant extent [71–73]. LDV-infected cells were recognized by the presence of viral RNA or viral proteins in infected cells (see Fig. 2A). Up to 70% of the peritoneal macrophages obtained from 1- to 2-week-old mice of various strains can be productively infected by LDV, but between 2 and 5 weeks of age the proportion of permissive macrophages in the peritoneum decreases to a lower level [37, 56]. The levels of permissive macrophages in the peritoneum decrease to 20–40% in adult mice that are maintained under pathogen-free conditions, but to 0–15% when not maintained under such conditions, most likely as a consequence of an inapparent infection with mouse hepatitis virus ([25] and Even and Plagemann, unpublished data). Replication of LDV in permissive macrophages is rapid and cytocidal [56, 72]. Viral RNA synthesis begins 3–4 h p.i. and maximum virus is produced by about 16 h p.i. with a yield of about 10^4 50% infectious units (ID_{50})/cell [73]. Cell death ensues soon thereafter [56]. Fluorescent antibody staining of infected macrophages and electron microscopic examination of sections of the infected cells have indicated that the production and accumulation of LDV proteins and of virus maturation are largely restricted to perinuclear regions (Fig. 2A,B, respectively). The first sign of infection of macrophages is the formation of clusters of free and membrane-bound polyribosomes in this region as well as of double-membrane vesicles, 100–300 nm in diameter (see Fig. 2B), at 3–4 h p.i. [58, 72]. This is followed by an abundant formation of nucleocapsid cores which mature into virions by budding into single-membrane vesicles generally in areas adjacent to the clusters of accumulating double-membrane vesicles and close to Golgi membranes (Fig. 2B).

   Our present hypothesis concerning the synthesis of the virion envelope proteins and of virus maturation is as follows: M/VP-2 and VP-3P are synthesized on rough endoplasmic reticulum (ER) and the signal peptide of VP-3P is removed. In the lumen of the ER vesicles, VP-3P becomes core glycosylated and disulfide bonded to M/VP-2 (Fig. 1B). The protein heterodimers then traverse the Golgi complex whereby the oligosaccharide side chains are processed and converted to complex oligosaccharides. The virion nucleocapsids then bud into the vesicles carrying the VP-3P/VP-2 heterodimers as they are released from the trans cisternae of the Golgi complex (see Fig. 2B) or after the vesicles have fused with each other or with other cytoplasmic membranes. The unusually basic endodomains of both M/VP-2 and VP-3P of these viruses (pI 9.4–11.9) may play an important role in virus maturation. They might interact with virion RNA, which is probably accessible in nucleocapsid cores, since the virion RNA is highly sensitive to degradation by RNase in isolated nucleocapsids [7].

   The ORF 2 proteins (see Fig. 1B) are probably synthesized via the same route as the VP-3/VP-2 heterodimers. Work with EAV has shown that G_S forms disulfide-linked homodimers [23] and is present in virions only at a density of 1–2% of that of the G_L/M heterodimers, while the two are present in similar amounts in infected cells [22]. The potential function of the ORF 2 protein present in such low density in the envelope in virus replication is unknown. The origin of the double-membrane vesicles and their potential function in virus replication are also unknown [58, 59]. The mode of virus release from the cells most likely occurs via movement of virus-containing vesicles to the plasma membrane followed by exocytosis.

Numerous cell lines, including many mouse macrophage cell lines, have been shown not to support LDV replication [56, 59, 72]. Apparently they lack a surface component that can function as an LDV receptor since resistant cell lines can be productively transfected with LDV RNA [38] or infected via the mouse hepatitis virus (MHV) receptor by pseudotype virions between MHV and LDV [25]. LDV infection of permissive mouse macrophages seems to involve receptor-mediated endocytosis [42], probably via a receptor that is expressed only at a specific stage of differentiation, but the identity of this receptor is unknown [9, 59]. Because of the lack of a permissive cell line, LDV can only be quantitated by an endpoint dilution assay in mice which is based on the increase in plasma LDH activity that accompanies LDV infection of mice [59].

In mice too, macrophages appear to be the primary or sole type of cell that supports LDV replication. Immunocytochemical staining [37, 61] and in situ hybridization [4, 66] have shown that at 1 day p.i. LDV-infected cells are present in practically all tissues, especially in macrophage-rich locations. For example, the greatest numbers of infected cells are present in lymph nodes, spleen, skin, lung and the leptomeninges of the central nervous system (CNS) [4]. In lymph nodes, LDV-infected cells are located in the marginal zone, the paracortex and the capsule (Fig. 3A,B). In the spleen, they are mainly located in the marginal zone between red and white pulp (Fig. 3C,D). In the CNS, LDV-infected cells are restricted to the leptomeninges [4].

Upon primary infection of a mouse, LDV productively infects all the permissive macrophages (estimated at about 10^6 cells/mouse) within the first day p.i. [50, 59, 71, 73]. Plasma LDV titers reach up to 10^{10} ID$_{50}$/ml 1 day p.i. (Fig. 4). Thereafter, plasma LDV titers decrease progressively during the next 3–4 weeks to a constant level which varies with the mouse strain (10^4–10^6 ID$_{50}$/ml; Fig. 4), but persists for the life of the mouse along with elevated plasma LDH activity [59]. By 2–3 days p.i., practically all permissive cells have been destroyed. The persistent infection is then maintained by replication of LDV in new permissive macrophages that are continuously, but only slowly, regenerated from apparently nonpermissive precursor cells [59, 71, 72]. Recent in situ hybridization studies have consistently detected a low but about constant number of LDV-infected cells in the liver, the paracortex of lymph nodes, the marginal zone of the spleen and in testes of persistently infected mice up to 90 days p.i. [66]. Another tissue in which LDV replication persists seems to be the thymus ([65] and below). These seem to be the primary organs in which new LDV-permissive macrophages arise or localize since no infected cells were detected in any other tissue of persistently infected mice [66].

In addition to the low numbers of LDV-infected cells in lymph nodes and spleen, in situ hybridization has demonstrated the accumulation of large amounts of LDV RNA in germinal centers of these tissues (e.g., Fig. 3E,F) which begin to be formed within 3 days p.i. [67]. The diffuse distribution of the LDV RNA suggests that it is associated with LDV virions that become trapped, perhaps mainly as immune complexes (see below), by the follicular dendritic cells (FDC) present in the light zone of the germinal centers (Fig. 3E,F). The formation of the germinal centers and accumulation of LDV virions within them coincide with the formation of anti-LDV antibodies and the polyclonal activation of B cells that is reflected by an elevation of plasma IgG2a and the appearance of low molecular weight immune complexes in the circulation (Fig. 4 and see below).
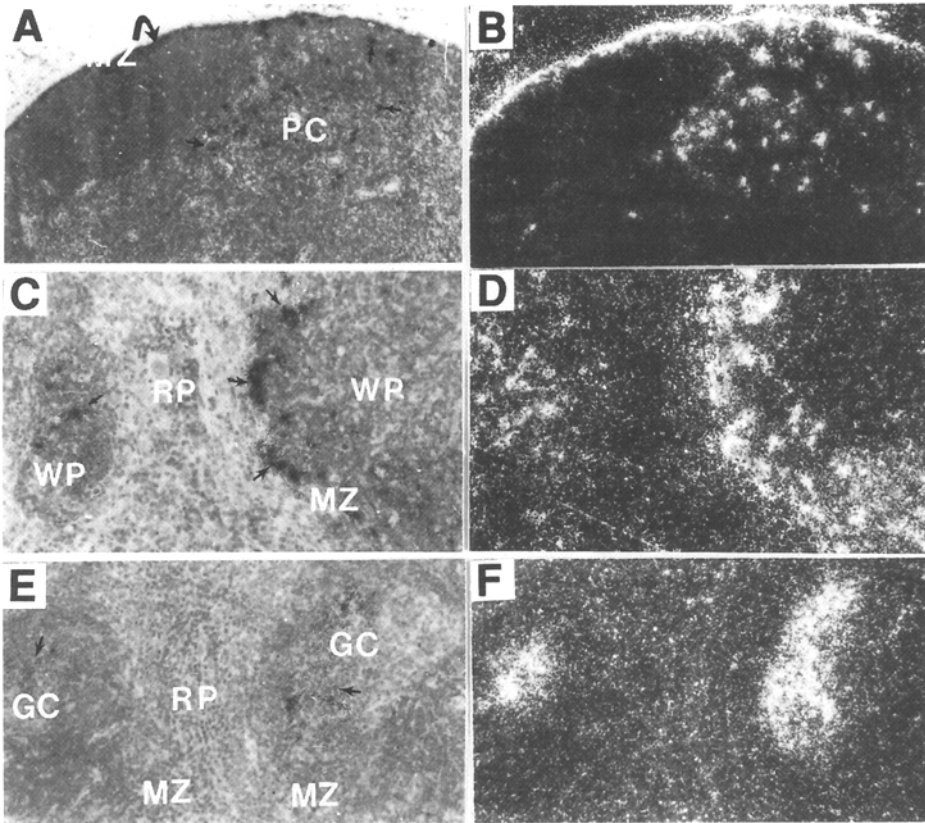
Fig. 3. Distribution of LDV RNA in sections of lymph nodes (**A, B**) and spleen (**C–F**) of 1-day LDV-infected (**A–D**) and 90-day LDV-infected (**E, F**) FVB mice. Sections of formalin-fixed, paraffin-embedded tissues were hybridized with a ^{35}S-labeled 437-bp cDNA probe (4-55) specific for the 3' end of the LDV-P genome [4, 66]. The sections were then washed, coated with photographic emulsion, exposed and stained with hematoxylin and eosin. *Arrows* indicate LDV-infected cells or accumulation of LDV RNA. **B, D, F** are dark-field images of the same microscopic fields as the bright field images shown in **A, C** and **E** (*MZ* marginal zone, *PC* paracortex, *RP, WP* red and white pulp, respectively, *GC* germinal center), **A–F** ×400

Failure of anti-LDV immune responses to control LDV replication

LDV replication in macrophages seems impervious to all host defenses that are normally operating in controlling virus infections. Failure of the immune system to control LDV infection is indicated by the finding that the time course and level of viremia in nude mice, which fail to mount an anti-LDV immune response, as well as in mice that have been rendered immunotolerant by infection as newborns, are the same as in their immunocompetent littermates [55, 65]. Immunotolerance seems to be maintained for the life of a mouse, probably as a result of continuous generation of LDV antigens in the thymus ([65] and see above). During the persistent phase of infection, LDV replication and thus viremia seem to be controlled by the rate of regeneration of permissive macrophages rather than the immune response [55, 65]. This conclusion is further supported by the finding that treatments that stimulate macrophage generation can enhance viremia during the persistent infection [65].
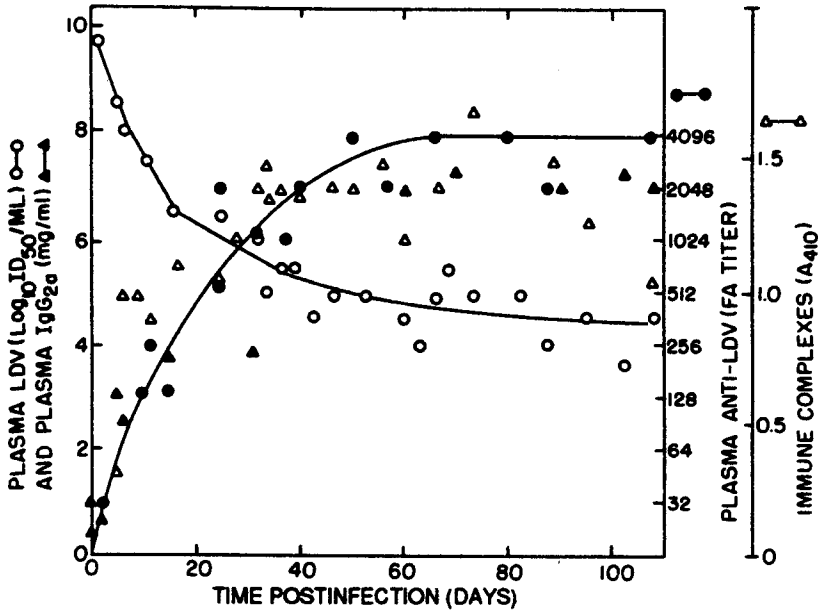
Fig. 4. Levels of infectious LDV, anti-LDV antibodies, IgG2a and hydrophobic low molecular weight immune complexes in plasma of 6-week-old BALB/c mice as a function of time after infection with LDV-P. A group of six mice was bled at the indicated times post infection (p.i.) with LDV-P. The pooled plasma was assayed for infectious LDV (o–o) by an endpoint dilution assay in mice [59], for anti-LDV antibodies (●–●) by a fluorescent antibody titration assay [12], for total IgG2a (▲–▲) by capture ELISA [44] and for immune complexes (△–△) that bind to ELISA plates not coated with antigen [12, 36]. Composed from data in [44] and [55]

The question arises as to how continuous LDV replication escapes all host defenses. The reasons have not been entirely resolved, but none of the normal defenses that play a role in the protection and/or recovery from viral infections, such as interferon-α/β (IFN-α/β), antiviral antibodies and cytotoxic T cells (CTLs) are effective in controlling LDV infection. Large amounts of IFN-α/β become transiently generated in infected mice during the 1st day p.i., being probably produced by the large number of macrophages that become infected during this time period [59]. However, LDV replication in macrophages is relatively resistant to IFN-α/β [71]. Furthermore, no IFN-α/β is detectable in the circulation of persistently infected mice; thus the amounts produced seem too low to have any effect on LDV replication.

Antiviral antibodies normally play a role in antiviral defenses by neutralizing viral infectivity, antibody-dependent complement lysis of virions and antibody-dependent cell-mediated cytotoxicity (ADCC). However, anti-LDV antibodies, though efficiently generated in high titers, are ineffectual in preventing or suppressing LDV infections. Anti-LDV antibodies begin to appear 4–5 days p.i. and reach maximum levels 3–5 weeks p.i. (Fig. 4). These antibodies are directed mainly to the primary envelope glycoprotein (VP-3P) and are mainly of IgG2a, IgG2b and IgG3 isotypes [17, 36, 44]. Early antibodies fail to neutralize LDV infectivity, neutralization being measured by incubation of virions with antibodies in vitro and then titrating residual infectivity in mice [59]. Antibodies that neutralize LDV under these conditions appear in mice only about 1 month p.i. and they neutralize LDV infectivity in vitro relatively poorly

[31, 59, 60, 67, 68]. The latter is also the case for most monoclonal antibodies to the envelope glycoprotein VP-3P [17, 31]. Although certain anti-VP-3P antibodies can neutralize LDV infectivity in vitro, as mentioned already, they have no significant effect on the course of the infection in vivo (Fig. 4). This finding also indicates that ADCC plays no role in controlling LDV replication. Furthermore, passive immunization with polyclonal or monoclonal neutralizing anti-VP-3P antibodies does not provide any protection to mice from LDV infection or affect the level of viremia in persistently infected mice [31].

In vitro neutralization of LDV is only observed at high antibody concentrations [31, 60] and is associated with an increase in sedimentation rate of the virions by $\geq 40\,S$ [60.] These results indicate that binding of the anti-VP-3P antibodies to virions is inefficient and that in vitro neutralization requires the binding of multiple antibody molecules per virion. In addition, in vitro neutralization is not affected by complement, even though many of the anti-LDV polyclonal and monoclonal antibodies are of the IgG2a or IgG2b isotype, which efficiently bind complement [31].

It is unclear why anti-LDV antibodies are so inefficient in vitro, and even more so in vivo, in neutralizing LDV infectivity. This inefficiency could be related to the unique structures of the virion envelope proteins (Fig. 1B). Only 9 amino acids of M/VP-2 and about 30 amino acids of VP-3P seem to be exposed on the virion surface. The ectodomain of M/VP-2 seems too small to react with antibodies and overall the protein seems to possess low immunogenicity. No antibodies to it have been detected in infected mice [12, 27] or among several batteries of monoclonal antibodies [17, 31]. The ectodomain of VP-3P is also very small and probably possesses only a limited number of neutralizing epitopes; all five neutralizing monoclonal antibodies generated in one set of monoclonal antibodies reacted with a single epitope [31]. The limited size of the ectodomain may provide little space for interaction with antibodies which may be further impeded by large polylactosaminoglycan side chains and the disulfide linkage of the protein to M/VP-2 to form the virus attachment site. For example, it has been shown that the single polylactosaminoglycan on the NB protein of influenza B virus masks the N-terminal end of the protein from antibody recognition [76]. Nevertheless, throughout the persistent infection practically all virions in the circulation are associated with antibodies in immune complexes in which LDV infectivity can be partially neutralized by anti-mouse IgG [12, 17, 59] and which may become trapped by FDCs in germinal centers (see above). These virion-antibody immune complexes differ from the low molecular immune complexes formed in association with the polyclonal activation of B cells induced by LDV infection (see below).

The only situations in which anti-LDV antibodies inhibit LDV infection is in blocking transplacental LDV transmission and in the paralytic infection of C58 and AKR mice [32, 52]. In the latter and other mice that possess at least one endogenous replication-competent ecotropic murine leukemia virus (MuLV) and the Fv-1$^{n/n}$ genotype, expression of the ecotropic MuLV in CNS glial cells renders anterior horn neurons susceptible to cytocidal infection by neurovirulent strains of LDV [3]. How the ecotropic MuLV expression induces LDV permissiveness in anterior horn neurons and what makes them LDV permissive is unclear. One likely possibility is that LDV permissiveness of the motor neurons is due to expression of a novel surface component on these cells that can function as an alternate receptor for neurovirulent strains of LDV. Destruction of the motor neurons by LDV infection can lead to fatal paralysis 2–3 weeks p.i. [3, 4, 58]. However, both neutralizing and non-neutralizing

anti-LDV antibodies block the paralytic LDV infection, either by preventing LDV from entering the CNS or by blocking motor neuron infection or both [4, 32]. This motor neuron protection by anti-LDV antibodies is effected without interference with LDV replication in macrophages [32].

CTLs play a primary role in the recovery from many viral infections by lysing infected cells presenting a viral peptide on their class I MHC molecules. Recently it has been demonstrated that N/VP-1-specific CTLs become rapidly generated in LDV-infected mice but that they have disappeared by 30 days p.i. [26]. Two sets of target cells were used: 7-h LDV-infected, cultured macrophages from pathogen-free B10.A mice (H-2^a; K^bD^d) and 3T3-NIH cells (H-2^s) that had been infected with a MuLV vector (LXSN) carrying the LDV ORF 7 encoding the N protein (3T3-29-4 and 3T3-29-5). Spleen cells isolated from B10.A mice or Swiss mice (H-2^s) at any time p.i. with LDV failed to lyse these target cells. However, when the spleen cells from 7-day LDV-infected B10.A or Swiss mice were exposed to LDV-infected B10.A macrophages or 3T3-29-4/5 cells, respectively, and then cultured in presence of IL-2, CD8$^+$ T cells became amplified and lysed the target cells in a H-2-specific manner (Fig. 5). For example, the CTLs from Swiss mice lysed the 3T3-29-4 and 3T3-29-5 cells, but not the LDV-infected B10.A macrophages, whereas the opposite was the case for the CTLs from B10.A mice. CTLs from Swiss x B10.A F1 hybrid mice (H-$2^{s/a}$) lysed both target cells, whereas 3T3-28-1 cells, which were infected with a MuLV vector carrying ORF 7 in the wrong orientation for expression, as well as uninfected macrophages from B10.A mice were not lysed (Fig. 5). CTLs were not yet detectable in either B10.A or Swiss mice at 4 days p.i. CTLs became amplified upon culture of spleen cells from 14- and 21-day-infected mice, but less efficiently than from spleens of 7-day-infected mice and by 30 days p.i. no CTL precursors were detectable anymore [26].

The reason for the disappearance of the CTLs is probably clonal exhaustion. Regardless, even when CTLs are present they have little or no effect on LDV replication in mice. This was indicated, as discussed already, by the finding that LDV viremia is the same in nude mice as in their immunocompetent littermates. Lack of CTL effectiveness was confirmed by inducing primary and secondary CTL responses in Swiss mice by injection of 3T3-29-4 cells that express LDV N/VP-1 and then challenging the mice with LDV [26]. Injection of the Swiss mice with 3T3-29-4, but not 3T3-28-1, cells induced a primary CTL response by 7 days after injection, which had subsided by 28 days after injection of the cells. A transient secondary CTL response was induced by a second injection of 3T3-29-4 cells at that time. Injection of such mice either during the primary or secondary CTL response with LDV reduced LDV replication only by about one $\log_{10}$ and only transiently [26].

The constant level of viremia during the persistent phase of infection (Fig. 4) represents a steady-state between the rates of LDV production and "clearance" of infectious virus from the circulation. LDV production depends on continuous rounds of de novo infection of permissive cells since a productively infected cell dies within 1 day p.i. The rate of LDV production is a function of the rate of generation of new LDV-permissive macrophages. The mechanism of loss of infectious LDV from the circulation is less clear. However, since anti-LDV immune responses do not seem to play a role in LDV clearance, loss of infectious LDV is probably mainly a consequence of physical inactivation. This conclusion is supported by the findings that the initial loss of infectious virus between 1 and about 15 days p.i. begins before the mouse mounts significant anti-LDV immune responses, that it is a single-hit process (Fig. 4)
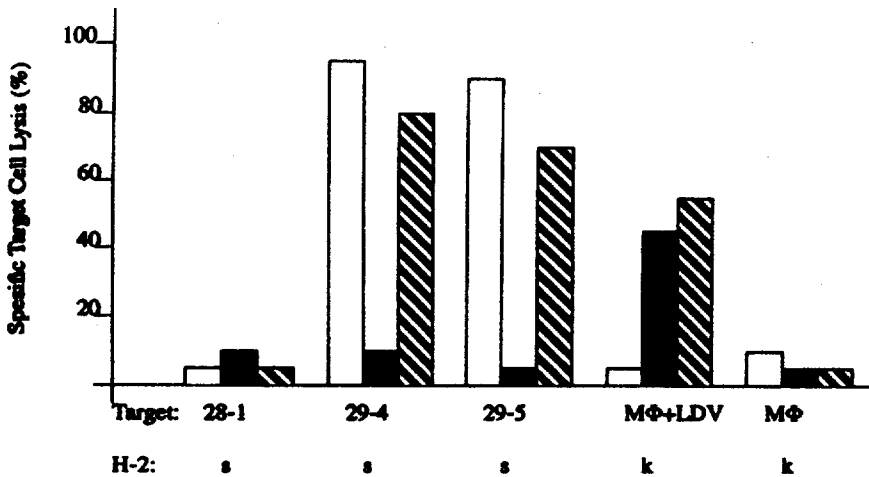
Fig. 5. Cytotoxic T lymphocyte (CTL) activity of spleen cells from 7-day LDV-infected Swiss (H-2^s; □), B10.A (H-2^a; ■) and Swiss x B10.A F1 hybrid mice (H-2$^{s/a}$; ◨). The spleen cells (10^6/ml) were incubated overnight with LDV VP-1-expressing 3T3 cells (H-2^s) or LDV-infected macrophages from B10.A mice and propagated with IL-2 for 5 days. The cells were then incubated at a ratio of 25:1 with the indicated target cells (for description see text) for 5 h and the culture fluid was assayed for LDH activity. Percent specific target cell lysis was calculated as [($\overline{X}$ − CTL-Sp–TAR-Sp)/(TAR-Max − TAR-SP)]× 100, where $\overline{X}$ = mean of total LDH released in three cultures; CTL-Sp = LDH released spontaneously from effector spleen cells; TAR-SP = LDH released spontaneously from target cells; TAR-Max = total LDH activity of target cells. Data are from [26]

and that the half-life of LDV loss of 10–12 h is similar to that observed for LDV inactivation in vitro at 37°C [67]. At this early stage of infection the production of LDV by infection of newly generated permissive macrophages is insignificant relative to the overall plasma LDV titer. However, eventually a steady state between virus production and physical inactivation is reached. On the basis of these considerations, we estimated that the infection of 100–1000 new permissive macrophages/day seems sufficient to account for the observed level of persistent viremia (Fig. 4).

Effects of LDV infection on the host immune system

LDV infection of mice has both short-term and long-term effects on the host immune system, but none seems to have a significant effect on the health of the host. The initial infection results in a transient suppression of cellular immune responses [33, 35, 59] as reflected by increased survival of tissue and tumor transplants and reduced delayed-type hypersensitivity, lipopolysaccharide-induced inflammatory responses and experimental allergic encephalitis induced in mice by injection of myelin basic protein. Suppression of cellular immune responses correlates in time with a transient 30–40% decrease in thymus weight [67] and impaired proliferative responses of spleen T cells in vitro to concanavalin A (Con A; Fig. 6) and anti-CD3 antibodies and an impaired ability of the T cells to produce IL-2, effects that are normally associated with T cell anergy ([44] and unpublished data). The mechanism by which these effects are caused by LDV infection has not been elucidated. They may have

multiple causes. Some effects may be triggered by cytokines released from the large number of macrophages that become initially productively infected. Also, IFN-γ has been implicated as a factor in the suppression of some immune functions [33].

Nitric oxide (NO) produced by activated residual macrophages could be one factor suppressing T cells, but our results do not support this suggestion. We found that the residual peritoneal macrophages present in 3-day LDV-infected mice possess an about sevenfold-increased capacity to produce NO but express this capacity only when cultured with IFN-γ [64]. The macrophages seem only partially activated because insufficient IFN-γ is generated at this stage of the infection (see below) to induce NO production in these cells. Even later in infection, the amount of IFN-γ may be too low or is too localized to induce significant NO production in macrophages.

In contrast to the transient suppression of cellular immune responses during the 1st week p.i., humoral antibody responses to both T cell-dependent and -independent antigens are enhanced during this time period [50, 58, 59]. These enhanced antibody responses to injected antigens correlate with a nonspecific polyclonal activation of B cells which progresses into an enhanced and persistent production of polyclonal IgG2a, or in some mice, IgG2b [16, 18, 36, 44].

The correlation between the formation of LDV antibodies, the polyclonal acti-vation of B cells (Fig. 4), increased specific antibody responses, the generation of germinal centers and accumulation of LDV in these germinal centers suggest that these observations are causally related. They are also associated with hyperplasia of lymph nodes and splenomegaly [67]. The latter seem to be partly due to trapping of lymphocytes and erythrocytes [39] but also probably partly to the proliferation of B cells. Both the anti-LDV antibody response and the polyclonal activation of B cells are T cell-dependent processes [12, 18, 44]. Furthermore, the polyclonal activation of B cells occurs only in mice that are actively infected by LDV and not in mice immu-nized with inactivated LDV [12]. The polyclonal activation of B cells is apparent by spontaneous proliferation and IgG2a production by spleen B cells from LDV-infected mice [18, 66]. When isolated from T cell-depleted mice, the B cells also proliferate spontaneously but fail to produce IgG2a unless cultured in the presence of IFN-γ [18].

In agreement with current models of B cell responses [40, 45, 46], the combined data summarized above suggest the occurrence of the following related events during the first few days p.i. with LDV. An LDV protein may function as a general B cell mitogen which perhaps in combination with IL-1 and IL-6 produced by LDV-infected macrophages activates B cells in the spleen and lymph nodes [18]. The LDV antigens, being produced in large amounts in the spleen and lymph nodes in close association with follicles (see Fig. 3C,D), induce the formation of germinal centers in which LDV becomes trapped by FDCs. Additional B cells become activated by the trapped LDV antigens, process the LDV antigens and present them to T cells which become activated. Interaction of the T cells with LDV-specific B cells results in production of anti-LDV antibodies, whereas the polyclonally activated B cells differentiate into IgG2a-producing plasma cells in response to IFN-γ produced by activated Th1 cells. IFN-γ is also probably produced by LDV-specific CD8$^+$ CTLs that are rapidly induced in the lymphoidal tissues (see above). In agreement with this scenario, spleen cells from LDV-infected mice have been shown to produce increased amounts of IFN-γ in response to Con A (Fig. 7). Not only do they produce increased amounts of IFN-γ, but the latent period of about 15 h before production of IFN-γ begins after exposure to Con A observed with spleen cells from uninfected mice is abolished in spleen
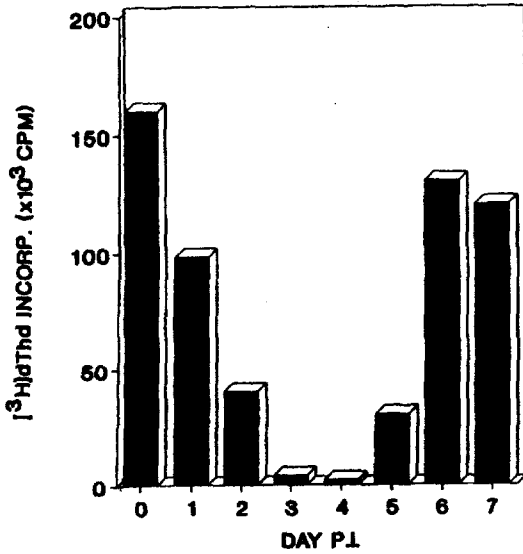
Fig. 6. Effect of LDV infection of C58/M mice on concanavalin A (Con A)-induced proliferation by their spleen cells in vitro. Spleen cells were harvested from groups of two mice at the indicated times p.i. with LDV and cultured in triplicate in 96-well tissue culture plates (10^6 cells in $100\,\mu l$ of medium/well). The wells'were supplemented with $2\,\mu g$ Con A/ml. After 2 days of incubation at 37°C, $1\,\mu$Ci [^{3}H]thymidine was added to each well and after another 15 h of incubation at 37°C, the cells were analyzed for radioactivity. Unpublished data of Rowland and Plagemann

cells from LDV-infected mice (Fig. 7). This finding suggests that the spleen cells from LDV-infected mice contain preformed IFN-γ mRNA. This conclusion has been confirmed by Northern hybridization analyses of total spleen RNA. The spleens from 3-week LDV-infected mice contained IFN-γ mRNA, whereas none was detected in the spleens from uninfected mice (unpublished data). The opposite was the case for IL-2 mRNA (unpublished data).

The polyclonal activation of B cells in infected mice is associated with the formation of autoantibodies exhibiting a wide range of specificities [10, 59, 75] as well as the appearance of low levels of IgG2a (or IgG2b)-containing hydrophobic immune complexes of 150–300 kDa [12, 36, 65]. The latter most likely consist of autoantibodies and their cellular antigens since they contain IgG of the same isotype (IgG2a or IgG2b) that is produced polyclonally. They do not represent complexes between LDV antigens and their antibodies since they are produced in mice in which no anti-LDV antibodies are produced [65].

During the persistent phase of infection with LDV, both primary and secondary humoral antibody responses are slightly suppressed [50, 62]. This effect could be due to IFN-γ suppression of Th2 cell functions and cytokine production.
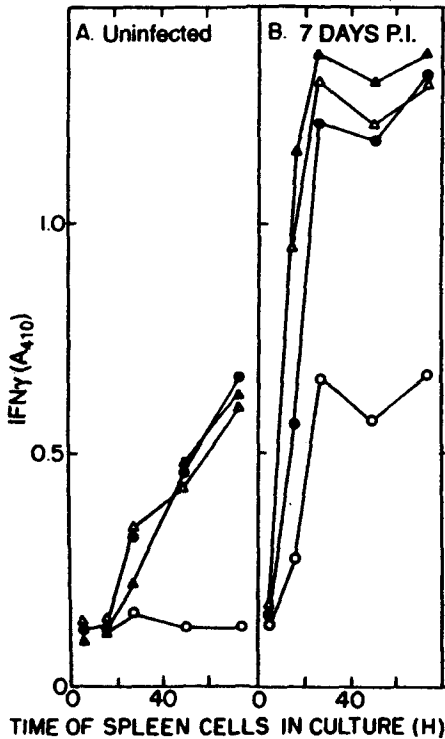
Fig. 7. IFN-γ production by spleen cells from uninfected and 7-day LDV-infected BALB/C mice after exposure to various concentrations of Con A. Spleen cells were cultured in 24-well tissue culture plates (5×10^6 cells in 2 ml of RPMI-10) with 0 (o-o), 1.5 (●-●), 3 ($\triangle$-$\triangle$) or 6 ($\blacktriangle$-$\blacktriangle$) μg Con A/ml. At various times of incubation at 37°C, small aliquots of culture fluid were removed and assayed by capture ELISA for IFN-γ concentration (A_{410}). Unpublished data of Hu, Rowland and Plagemann

Persistence in nature and transmission

The two LDV strains whose genomes have been sequenced have been isolated from laboratory mice that carried different LDV-contaminated, transplantable mouse tumors [59, 67]. The origins of these LDVs is unknown, but they probably originated from LDVs endemic in wild house mouse (*Mus musculus domesticus*) populations [67]. LDVs have been isolated from wild house mice in Europe, the USA and Australia [67]. However, not all mice in the wild are infected, probably because LDV transmission between mice is relatively inefficient, in spite of the life-long, relatively high viremia in infected mice and the secretion of the virus in urine, feces and saliva [59, 67]. Horizontal transmission between mice seems restricted by the mucosal barrier since infection of mice by LDV via oral, vaginal, rectal and ocular routes requires four to six orders of magnitude higher amounts of LDV than via injection [11], and no intranasal infection has been achieved (Plagemann and Cafruny, unpublished data). Horizontal transmission is observed in laboratory mice that are fighting and biting, but what role this route of transmission plays in the wild is unclear. The possibility that LDV is maintained in wild mouse populations by sexual transmission needs further study because LDV is probably continuously secreted via semen by persistently infected

males (see above). LDV is highly infectious when injected, regardless of the route of injection [11, 59, 67]; one mouse ID_{50} probably represents only a few virion particles. LDV is transmitted from mother to offspring transplacentally or via breast milk, but only if the mother has not mounted an anti-LDV immune response because anti-LDV antibodies block transplacental transmission of the virus and its release into milk [58].

Comparisons of LDV with other members of its family

LDV, EAV, PRRSV and SHFV are structurally indistinguishable as are their genome organization and expression. Also, the proteins of EAV and especially of PRRSV are closely related to those of LDV in size, amino acid sequence, and structure [58, 59] (see Fig. 1). These viruses share other properties: macrophages are their primary or sole host cell; their growth cycle, mode of replication and cytopathogenicity in these cells are comparable; and they all can establish persistent infections in their hosts [47, 53, 58, 59]. EAV and PRRSV are transmitted sexually via semen and transplacentally from mother to offspring. However, marked differences in pathogenicity exist. EAV and PRRSV similarly cause respiratory and reproductive failure in their hosts. In contrast, SHFV establishes asymptomatic persistent infections in several genera of African monkeys, just as LDV does in mice [59], but accidental transmission of SHFV to Asian macaque monkeys in monkey colonies results in fatal hemorrhagic fever. Transmission between macaque monkeys is via the respiratory route, just as is transmission of EAV and PRRSV in epizootics, whereas this route of transmission is not observed for LDV [59]. Furthermore, in contrast to persistent LDV infections, antiviral antibody levels are generally very low or nonexistent in African monkeys persistently infected with SHFV, and SHFV, EAV and PRRSV infections seem to be suppressed by antiviral immune responses [47, 53, 59]. In EAV and PRRSV infections, the generation of antiviral immune responses correlates with termination of viremia and vaccination is effective in protecting horses from EAV infection. The reasons for the differences in effectiveness of the host immune system in controlling EAV and PRRSV infections, but not LDV infections, are unclear. In any case, in spite of the antiviral immune responses, EAV and PRRSV replication may persist in some tissues, especially the testis. In fact, persistently infected stallions and boars are a common source of new infections [58]. Continuous virus replication in testes may be maintained by infection of newly generated permissive macrophages just as already described for LDV.

Conclusions

LDV contradicts all commonly held views about mechanisms of virus persistence, namely that persistence is primarily associated with noncytopathic viruses, or the selection of immune escape variants or other mutants, or a decrease in expression of certain viral proteins by infected cells, or replication in "immune-privileged sites", or a general suppression of the host immune system, etc. [1, 2, 5, 54, 77, 78]. LDV is a highly cytocidal virus that invariably establishes a life-long, viremic, persistence in mice, in spite of normal anti-viral immune responses.

One secret of LDV's success in persistence is its specificity for a renewable, nonessential population of cells that is continuously regenerated, namely a subpopu-

lation of macrophages. Since the continuous destruction of these cells is not associated with any obvious health effects, this macrophage population seems nonessential to the well-being of its host. The only function identified for this subpopulation of macrophages is clearance of the muscle type of LDH and some other enzymes [59, 67, 68]. Furthermore, the effects of LDV infection on the host immune system, namely the polyclonal activation of B cells and its associated production of autoantibodies, and the slight impairment of primary and secondary antibody responses also do not seem to be severe enough to cause any clinical consequences.

But how does LDV replication in macrophages escape all host defenses? Persistence is not dependent on the seletion of immune escape variants or other mutants ([58] and Palmer, Even and Plagemann, unpublished results). Also, LDV replication is not restricted to immune-privileged sites [5]. LDV replication persists in the liver, lymphoidal tissues and testis [66]. Only the latter could be considered a site not readily accessible to immune surveillance.

Most likely, resistance of LDV replication to antiviral immune responses is related to the unique structure of its envelope proteins and the production of large quantities of viral antigens. High titers of anti-LDV antibodies are generated in infected mice but they neutralize LDV infectivity only very inefficiently and, even though the antiviral antibodies are mainly of the IgG2a and IgG2b isotypes, they do not mediate complement lyses of virions [31]. Interaction of the antibodies and complement with the VP-3/VP-2 heterodimers in the viral envelope may be impeded by the exposure of only very short peptide segments of these proteins at the envelope surface and the presence of large oligosaccharide side chains. Furthermore, since LDV maturation is restricted to intracytoplasmic cisternae [59, 71], the question arises of whether any of the viral proteins are available on the surface of infected cells for ADCC.

CTLs also fail to control LDV replication. Altough CTLs specific for N/VP-1 are rapidly generated, these have disappeared by 30 days p.i. [26]. The reasons for this loss are unknown, but high-dose clonal exhaustion [41, 51, 77, 78] is a reasonable possibility since, regardless of the infectious dose, large amounts of LDV proteins are present in all the lymphoidal tissues at the time of the induction of the CTL response. Furthermore, after exhaustion of CTLs in the periphery, continuous replication of LDV in the thymus [65] assures that the mice become permanently immunologically tolerant with respect to LDV antigen-specific CTLs as a result of negative selection in the thymus. LDV might be a primary example for the effectiveness of a permanent clonal CTL deletion in adult animals under natural conditions of infection.

The presumed modes of transmission of LDV in nature and the events associated with its infection of mice are strikingly similar to those observed during the acute and asymptomatic phases of infection with human immunodeficiency virus (HIV) [24, 29, 74, 78]. These include: (1) primary inefficient transmission via sexual and transplacental routes but effective transmission via blood; (2) primary replication in renewable populations of lymphoidal cells with production of large amounts of virus after the initial infection of the host followed by persistent low level of viremia in spite of antiviral immune responses; (3) persistence, reflecting continuous rounds of productive, cytocidal infection of permissive cells [59, 74] and the rate of generation of permissive cells which may be the main factor in determining the level of virus production (in the case of HIV, the rate of activation of $CD4^+$ T cells to support a productive HIV replication might be the factor determining the rate of virus production and the progression of the disease); (4) rapid antibody formation but delayed production of neutralizing antibodies with limited neutralizing capacity; (5) rapid but

transient generation of virus-specific CTLs; and (6) accumulation of large amounts of virus in newly formed germinal centers in the spleen and lymph nodes concomitant with an initiation of a permanent polyclonal activation of B cells resulting in an elevation of plasma IgG2a.

The events described under points 2–6 might be generally associated with natural viremic persistent virus infections. Such persistent viruses, by necessity, have evolved properties that allow them to escape all host defenses and control of their infection by immunological processes is, therefore, difficult, if not impossible. Prevention of infection and chemotherapy may be the only approaches available to combat such virus infections.

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