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LACTATE DEHYDROGENASE-ELEVATING VIRUS, EQUINE ARTERITIS VIRUS, AND SIMIAN HEMORRHAGIC FEVER VIRUS: A NEW GROUP OF POSITIVE-STRAND RNA VIRUSES

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I. Introduction

The last comprehensive reviews of nonarbo togaviruses (Brinton, 1980; Horzinek, 1981) included discussions on pestiviruses, rubella

virus, lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), cell fusion agent, and nonarboflaviviruses. The inclusion of all these viruses in the family Togaviridae was largely based on similarities in morphological and physical/chemical properties of these viruses and in the sizes and polarities of their genomes. All are spherical, enveloped viruses of similar size (between 40 and 60 nm in diameter) with a nucleocapsid core 25–35 nm in diameter and, as far as known, icosahedral symmetry containing a single molecule of positive-strand RNA of $(4–6) \times 10^6$ Da (Westaway *et al.*, 1985a, b; 1986) (Table I). In the intervening years considerable new information on the replication strategies of these viruses and the structure and organization of their genomes has become available, which has led to the reclassification or suggestions for reclassification of some of them. The *Flavivirus* genus has been elevated to the status of family, the Flaviviridae (Westaway *et al.*, 1985b). The pestiviruses, though unrelated to the flaviviruses serologically and only remotely in amino acid and nucleotide sequence, have been found to exhibit a genome organization and replication strategy resembling those of the flaviviruses (Collett *et al.*, 1988, 1989), and have thus been recently reclassified as a genus within the Flaviviridae (Moennig and Plagemann, 1991). The newly discovered togalike virus hepatitis C virus (HepCV; Choo *et al.*, 1989) exhibits a genome organization and other properties resembling those of the pestiviruses and flaviviruses (Table I) (Plagemann, 1991). Little new information is available concerning the cell fusion agent (Brinton, 1980; Horzinek, 1981). Sequence analysis of HepCV genomic RNA is in progress and initial results indicate that it is an insect virus also related to flaviviruses (V. Stollar, personal communication, 1991).

On the other hand, the replication strategy of EAV resembles that of the coronaviruses, involving a 3′-coterminal nested set of mRNAs (van Berlo *et al.*, 1982; Spaan *et al.*, 1990; den Boon *et al.*, 1991). Therefore, EAV has been suggested to be included in a virus superfamily along with coronaviruses and toroviruses (de Vries *et al.*, 1990; Spaan *et al.*, 1990; den Boon *et al.*, 1991). Recent evidence indicates that LDV not only resembles EAV in morphology, virion and genome size, and number and size of their structural proteins (see Table I) but also in genome organization and replication via a 3′-coterminal set of mRNAs (Godeny *et al.*, 1990; Kuo *et al.*, 1991). SHFV, although not fully characterized, exhibits properties resembling those of LDV and EAV and recent evidence suggests that it may possess the same genome organization as these viruses (Godeny and Brinton, 1991). The three viruses may therefore represent a new family of positive-strand RNA viruses and are

TABLE I

PHYSICOCHEMICAL PROPERTIES OF ALPHA AND RUBI TOGAVIRUSES, FLAVIVIRUSES, PESTIVIRUSES, AND UNGROUPED TOGA-LIKE VIRUSES[a]

Virus	Virion			Viral RNA			Structural proteins (kDa)		
								Envelope	
	Diameter (nm)	S	g/cm^3[b]	kb	3′ poly(A)	Subgenome mRNAs	NC[c]	Nonglycosylated	Glycosylated
Alphavirus	50–65	286	1.18–1.19	11–13	+	1	30–34	None	50–59 (El), 50–59 (E2)[d]
Rubivirus	60	240–350	1.19	~10	+	1	33–34	None	58–59 (El), 42–48 (E2)
Flavivirus	45–50	175–218	1.19–1.20	10–11	–	0	13–16	7–9	51–59
Pestivirus	50–60	140	1.12–1.15	12–15	–	0	27–28	None	gp44–48; gp25–33; gp53–55[e]
HepCV	40–60	200–280	1.09–1.11	~10	?	0	18–22	None	gp37; gp70[e]
EAV	60	224	1.15–1.17		+	6	12–14	17–18	28–42 (21)[f]
LDV	55	200, 230	1.13–1.14	12–13	+	7	14	18	24–44
SHFV	45–50	214	1.15	12–13	+	?	12	16–18	50

[a] Data for alphaviruses and rubiviruses are from Westaway *et al.* (1985a) and Horzinek (1981); for flaviviruses, from Westaway *et al.* (1985b); for pestiviruses and hepatitis C virus (HepCV), from Moennig and Plagemann (1991) and Plagemann (1991), respectively. For sources of data for the other viruses, see text.

[b] As determined by isopycnic centrifugation in sucrose or glycerol density gradients.

[c] NC, Nucleocapsid.

[d] May also contain a 10-kDa protein (E3).

[e] Tentative assignments.

[f] The viral origin of the 21-kDa protein has not been proved (W.J.M. Spaan, personal communication).

reviewed together. Emphasis will be on recent information concerning their molecular properties and pathogenesis *in vitro* and *in vivo* and on host immune responses to infections by these viruses.

II. Lactate Dehydrogenase-Elevating Virus

LDV was discovered 30 years ago as a contaminant of transplantable mouse tumors on the basis of causing a 5- to 10-fold increase in the level of lactate dehydrogenase (LDH) activity in the blood of infected mice (Riley *et al.*, 1960). This property has led to the generally accepted name of the virus, lactate dehydrogenase-elevating virus, and is the basis of the only titration procedure available for the virus at present (Plagemann *et al.*, 1963; Notkins and Shochat, 1963). LDV has attracted considerable attention as a research model because of (1) its unique restricted host specificity for a subpopulation of macrophages, (2) the lifelong persistent infection it invariably establishes in all mouse strains, which is asymptomatic except for subtle effects on the host immune system, and (3) its ability to cause under a multifactorial setting a generally fatal motor neuron disease (age-dependent poliomyelitis, ADPM) in certain mouse strains. LDV has also gained notoriety as a common contaminant of transplantable mouse tumors (Riley, 1974; Riley *et al.*, 1978). General aspects of LDV research have been covered in several previous reviews (Rowson and Mahy, 1975, 1985; Brinton, 1980, 1982, 1986; Cafruny, 1989). Other reviews have specifically dealt with ADPM caused by LDV (Murphy *et al.*, 1980, 1983, 1987; Contag *et al.*, 1989). The present review concentrates on recent findings dealing with the mechanism of LDV persistence, pathogenesis, effects on the host immune system, and molecular properties.

A. Acute and Persistent Infection of Mice

1. Virological Aspects

a. Species and Host Cell Specificities and Viremia in Mice. LDV readily infects wild mice and all strains of laboratory mice of the species *Mus musculus* and also of one other mouse species, *Mus caroli* (Rowson and Mahy, 1975, 1985). Attempts to infect other species, such as rats, hamsters, guinea pigs, and rabbits, with LDV have been negative (Rowson and Mahy, 1985). LDV is titrated by an end-point dilution assay in mice (Plagemann *et al.*, 1963; Rowson and Mahy, 1975). Groups of mice are injected intraperitoneally (i.p.) with 10-fold dilu-

tions of virus material, the mice are bled 4 days postinjection (when maximum plasma LDH elevation is attained), and their plasma is assayed for LDH activity. A 5- to 10-fold elevation in LDH activity above normal is taken as indication of an LDV infection. The dose at which 50% of the mice are infected (ID_{50}) is calculated by the method of Reed and Muench.

On infection of a mouse of any strain, LDV titers in plasma begin to increase about 5 to 10 hr postinfection and reach a maximum of 10^9–10^{10} ID_{50}/ml 1–2 days postinjection (Rowson and Mahy, 1975, 1985) (Fig. 1). Thereafter, plasma LDV titers decrease progressively over the next 3–4 weeks to a constant level of 10^4–10^5 ID_{50}/ml. This level of viremia persists for the rest of the life of the mouse along with elevated plasma levels of LDH activity.

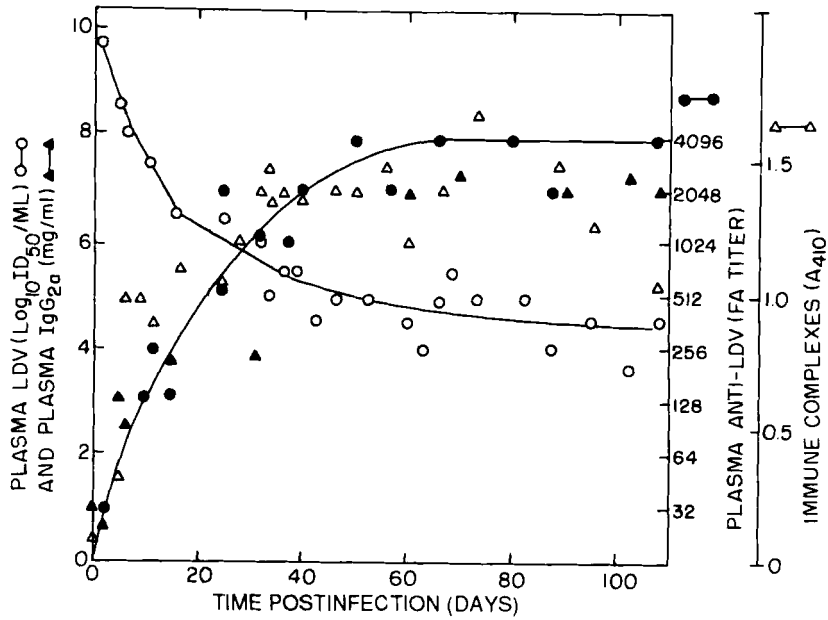

FIG. 1. Levels of infectious LDV, anti-LDV antibodies, IgG2a and immune complexes in plasma of 6-week-old BALB/c mice as function of time postinfection with LDV. A group of six mice was bled at the indicated times postinfection with LDV-P; the pooled plasma was assayed for infectious LDV by an end-point dilution titration in mice (○), for anti-LDV antibodies by an indirect fluorescent antibody titration assay (●), for total IgG_{2a} by ELISA (▲), and for immune complexes that bind to high-affinity protein-binding ELISA plates not coated with any antigen (△). [Data compiled from studies by Onyekaba *et al* (1989a) and Li *et al* (1990).]

This pattern of LDV viremia is explained by the restricted host specificity of LDV for a specific subpopulation of macrophages. LDV replicates in primary cultures of resident macrophages from the peritoneum, spleen, bone marrow, and probably liver and lung, but not in primary cultures of activated macrophages or peripheral monocytes (Rowson and Mahy, 1985; Kowalchyk and Plagemann, 1985). Up to 80% of the peritoneal macrophages cultured from 1- to 2-week-old mice support the productive infection by LDV as indicated by LDV RNA and protein synthesis in these cells (Inada and Mims, 1985b; Onyekaba *et al.*, 1989b). However, between 2 and 5 weeks of age the proportion of permissive resident macrophages in the peritoneum of mice decreases to 5–10%, which is the level typically observed for adult mice of any strain (Tong *et al.*, 1977; Onyekaba *et al.*, 1989b). The reason for the change in LDV permissiveness of peritoneal macrophages with age of the animal is unknown, but it probably reflects a decrease in the proportion of cells that express a surface protein that acts as LDV receptor (see later), because the efficiency of infecting individual permissive macrophages from mice of different ages *in vitro* is about the same (Onyekaba *et al.*, 1989b). The infection of macrophages *in vitro,* however, is relatively inefficient as compared to the infection of mice. Cultures of macrophages must be exposed to $\geq$100 *in vivo* ID_{50} of LDV/macrophage to achieve infection of all LDV-permissive macrophages in the population (Tong *et al.*, 1977; Kowalchyk and Plagemann, 1985; Onyekaba *et al.*, 1989b). The reasons for the low efficiency of infection of cultured peritoneal macrophages have not been explored. Cultured macrophages are partially in a state of activation and it seems possible that most virions entering these cells become degraded and that a high multiplicity of infection (m.o.i.) is required to allow a few virions to escape this process and establish a productive infection. On the other hand, cultured macrophages may only express low levels of LDV receptors (see later). A related possibility is that a subpopulation of macrophages or of another type of LDV-permissive cell exists in mice that is more susceptible to LDV infection than cells cultured from the peritoneum and other tissues, and that it is this population that becomes mainly infected initially upon i.p. injection of the virus. Significant LDV replication has only been observed in primary cultures of mouse macrophages and in primary mouse embyro cultures in which macrophages are probably the only cell type supporting LDV replication (Rowson and Mahy, 1975, 1985).

It has been reported that LDV replicates poorly in macrophages obtained from mouse hepatitis virus (MHV)-infected mice (Brinton, 1986). Because MHV is highly contagious and prevalent in mouse

colonies (Rowe *et al.*, 1963), the presence of low proportions of LDV-permissive cells in populations of peritoneal macrophages harvested from individual mice or different batches of mice (Stueckemann *et al.*, 1982a; Buxton *et al.*, 1988) could have been due to inapparent MHV infection of the donor mice. In these mice, LDV-permissive macrophages might have been destroyed by MHV infection, because macrophages are a major host cell for MHV and the majority of macrophages from mice of susceptible mouse strains can be infected by MHV (Smith *et al.*, 1984).

Numerous lines of transformed mouse macrophages have been examined for LDV permissiveness. None has been found to support LDV replication to a significant extent, whether the macrophages became transformed spontaneously or were transformed by simian virus 40, retroviruses or oncogenes, or by fusion with transformed cell lines (Stueckemann *et al.*, 1982a; Onyekaba *et al.*, 1989b; Rowson and Mahy, 1985). Only lines derived from peritoneal macrophages isolated from 1- to 2-week-old mice have been found to contain a low proportion of LDV-permissive cells (Onyekaba *et al.*, 1989b; see below). Various established lines of other mouse cells and of cells from other species also failed to support productive LDV infection. Thus LDV exhibits an extremely narrow species and host cell specificity.

b. Viral Macromolecular Synthesis in Infected Cells and Cytotoxicity. One-step growth experiments have shown that LDV replication in macrophages is rapid. Viral RNA and protein syntheses are first detected 3–4 hr postinfection and reach maximum rates at 6–10 hr postinfection (Stueckemann *et al.*, 1982a; Onyekaba *et al.*, 1989b). LDV replication in macrophages is highly cytocidal. In cultures of macrophages from 1- to 2-week-old mice, in which 70–80% of the macrophages become infected, all infected cells are killed and have disappeared within 24 hr postinfection (Inada and Mims, 1985b; Onyekaba *et al.*, 1989b). However, intact dead cells have not been observed in these cultures. The dead cells either rapidly and completely disintegrate or are rapidly phagocytozed by other still functional macrophages in the culture (Onyekaba *et al.*, 1989b). A similar disappearance of LDV-infected cells is observed in cultures of macrophages from adult mice (Stueckemann *et al.*, 1982a) and mouse embyro cultures (Oldstone *et* al., 1974), as well as in intact animals (Porter *et al.*, 1969; Inada and Mims, 1985b; Chan *et al.*, 1989). At 1 day postinjection LDV RNA is readily detectable in the spleens of infected adult mice by Northern blot and *in situ* hybridization (Contag and Plagemann, 1989; Chan *et al.*, 1989). LDV-infected cells (see Fig. 2) can be readily detected by electron microscopy in sections of spleens of infected mice 1 day postinjection

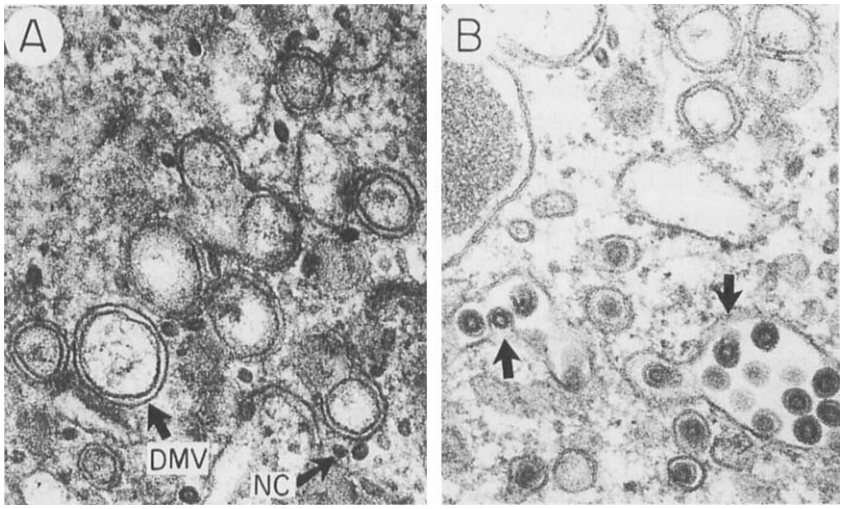

FIG. 2. Electron micrographs of thin selections of cultured mouse macrophages at 7 (A) or 12(B) hr postinfection with LDV-P. (A) DMV, Double-membrane vesicles; NC, free nucleocapsid cores. (B) Arrows indicate budding virions. Scale 1 cm = 0.1 μm. The micrographs were generated in a study by Stueckemann *et al.* (1982a).

(Stueckemann *et al.*, 1982a; Chan *et al.*, 1989) and LDV antigens are found by fluorescent antibody staining in practically all tissues, except the white and gray matter of the brain and spinal cord, but especially in the spleen and liver (Porter *et al.*, 1969; Inada and Mims, 1985b; Chan *et al.*, 1989). In the spleen, LDV-infected cells are primarily localized in the red pulp, the main location of macrophages in the spleen (Porter *et al.*, 1969; Chan *et al.*, 1989). However, both LDV RNA and antigens decrease in all tissues examined to below detectable levels by 2–3 days postinfection (Chan *et al.*, 1989; Inada and Mims, 1985b) and at this time no infected cells have been detected in spleen sections by electron microscopy (Stueckemann *et al.*, 1982a).

These results are explained as follows. Upon primary infection of a mouse, LDV rapidly establishes a productive, cytocidal infection in all permissive macrophages. It has been estimated that a mouse may possess about 10^6 permissive macrophages (Stueckemann *et al.*, 1982a). This estimate is based on the plasma LDV titers of mice 1 day postinfection and a viral yield of about 10^4 ID_{50}/cell observed in macrophage cultures. The LDV-permissive macrophages are probably lining the circulatory system, thus releasing progeny virus directly into the blood.

This location would explain the finding that plasma LDV titers are as high or higher than those observed in any isolated tissue (Plagemann *et al.*, 1963; Rowson and Mahy, 1985).

The destruction of all permissive macrophages after the primary infection of a mouse not only explains the disappearance of infected cells from all tissues, but also the decrease in plasma LDV titers observed during the next 3–4 weeks after the initial burst of LDV production (Onyekaba *et al.*, 1989a) (Fig. 1). LDV seems to persist in the animal by replicating in newly formed permissive macrophages as they arise from apparently nonpermissive precursor cells (Stueckemann *et al.*, 1982a; Onyekaba *et al.*, 1989a). New permissive macrophages probably arise continuously in the animal at a low rate. It has been estimated that the generation of 100–1000 permissive macrophages/day and their productive infection by LDV could account for the level of viremia observed in persistently infected mice (Stueckemann *et al.*, 1982a). The level of persistent viremia seems to reflect a balance between the production of LDV by infected macrophages and inactivation of the virus in the circulation, and seems little, if at all, affected by the anti-LDV immune response of the host (see below).

Although LDV infection of macrophages in primary cultures is highly cytocidal (Onyekaba *et al.*, 1989b; Inada and Mims, 1985b), such cultures are unsuitable for LDV titrations. In cultures of macrophages from adult mice, the proportion of LDV-permissive cells is too low to allow detection of their destruction (Stueckemann *et al.*, 1982a). Cell destruction is clearly apparent in LDV-infected cultures of macrophages isolated from 1- to 2-week-old mice but the establishment of these cultures is technically too difficult and too expensive for a routine assay. Furthermore, as pointed out already, infection of cultured macrophages is rather inefficient and LDV spreads very poorly, if at all, between permissive macrophages in these cultures (P. G. W. Plagemann, unpublished observations, 1991).

c. Plasma Enzyme Elevations in Infected Mice. The continuous destruction of LDV-permissive macrophages in infected mice probably accounts for the permanent elevation of LDH and other enzymes in their circulation. Only the level of muscle-type LDH (isozyme LDH-V), but not of the heart-type LDH (isozyme LDH-I), becomes elevated in LDV-infected mice (Plagemann *et al.*, 1963). The level of plasma isocitrate dehydrogenase (IDH) activity rises to a similar extent as that of LDH-V and the activities of malate dehydrogenase (MDH), glutathione reductase, phosphohexose isomerase (glucose-6-phosphate isomerase), and glutamic-oxalacetic transaminase (aspartate aminotransferase) become elevated after LDV infection to a lesser extent (Plagemann

et al., 1962; Rowson and Mahy, 1975). The plasma activities of aldolase, α-glycerophosphate dehydrogenase, glucose-6-phosphate dehydrogenase, alkaline and acid phosphatases, glutamic–pyruvic transaminase (alanine aminotransferase), and leucine aminopeptidase are not affected by LDV infection (Plagemann *et al.*, 1962).

Blood levels of enzymes reflect a balance between their release from tissues and removal by scavenger cells, mainly macrophages (Brinton and Plagemann, 1983; Rowson and Mahy, 1975, 1985; Smit *et al.*, 1987). In rats, LDH-V is cleared by macrophages that are located primarily in the liver, spleen, and bone marrow (Sinke *et al.*, 1979; Smit *et al.*, 1987), and clearance seems to be mediated by receptor-mediated endocytosis and intralysosomal proteolysis (Smit *et al.*, 1987). Endocytosis of LDH-V by rat Kupffer cells *in vitro* exhibits saturation kinetics (K_m about 1 μM) and is competitively inhibited by malate and alcohol dehydrogenases, creatine kinase, and adenylate kinase (Smit *et al.*, 1987). The K_i of inhibition by these enzymes approximately correlated with their rate of clearance *in vivo* (Smit *et al.*, 1987). These enzymes are all cleared rapidly from the circulation. They all seem to interact with the same receptor on macrophages that mediate their clearance. In contrast, LDH-I, which belongs to a group of enzymes that are cleared much more slowly from the circulation than LDH-V, does not inhibit the binding of LDH-V to rat Kupffer cells (Smit *et al.*, 1987). Initially it was thought that the positive charge of the basic LDH-V (p*I* 8.5) is a major factor in determining its interaction with the macrophage receptor, because the clearance of LDH-V in mice is strongly retarded by acetylation of the enzyme (Wachsmuth and Klingmüller, 1978), and LDH-I, which does not bind to the receptor, is a highly acidic protein (p*I* 4.5–4.8). However, this conclusion has been questioned because acidic enzymes such as adenylate kinase (p*I* 6.1) and creatine kinase (p*I* 6.8) show a high affinity for the rat macrophage LDH-V receptor, whereas the very basic enzyme ribonuclease A (p*I* 9.5) does not (Smit *et al.*, 1987).

LDV infection has been found to greatly impair the clearance from the circulation of LDH-V but not of the less rapidly cleared LDH-I (Rowson and Mahy, 1975, 1985). This finding along with the fact that only LDH-V, but not LDV-I, becomes elevated in LDV-infected mice suggest that the subpopulation of LDV-permissive macrophages in mice plays a major role in clearing LDH-V and IDH from blood and that the elevation of these enzymes is mainly a consequence of the destruction of the permissive macrophages by LDV infection. This view has recently been strengthened by the finding that injection of mice with silica particles that are toxic to macrophages also causes a marked

increase in plasma LDH activity (Hayashi *et al.*, 1988). Silica particles also cause an impairment in the clearance of injected porcine LDH by mice but not of injected LDH-I (Hayashi *et al.*, 1988). However, silica injection and LDV infection cause an additive impairment in LDH-V clearance. This result suggests that LDV and silica affect different subpopulations of macrophages, which both clear LDH-V, and that the LDV-permissive macrophages are resistant to silica and, vice versa, that silica-susceptible macrophages are not permissive for LDV. This conclusion is supported by the finding that pretreatment of mice with silica slightly increased rather than decreased the production of LDV during the first 24 hr postinfection (DuBuy, 1975). Furthermore, it has been observed that the rate of clearance of porcine LDH-V differed up to 30% in four different inbred mouse strains (Hayashi *et al.*, 1988).

Because the elevation of the other enzymes in LDV-infected mice is less than observed for LDH-V and IDH, LDV-permissive macrophages may only represent a minor population mediating the clearance of these enzymes. Malate dehydrogenase interacts with the LDH-V receptor on rat macrophages (Smit *et al.*, 1987) and becomes, like LDH-V, elevated in plasma of LDV-infected mice (Rowson and Mahy, 1975). It would be of interest to determine whether this correlation extends, on the one hand, to the other enzymes that interact with the rat macrophage receptor and, on the other hand, to IDH, which is known to become elevated in plasma of LDV-infected mice. It has already been shown that asparaginase, MDH, and porcine LDH-V, but not porcine LDH-I, bind to mouse peritoneal and spleen macrophages. (Nakayama *et al.*, 1990). Of these enzymes, asparaginase bound most effectively. It also bound to the cells of one macrophage line (RAW 264.7), but not to those of another macrophage line (IC21) or to BALB/c 3T3 cells or nonadhering spleen cells (Nakayama *et al.*, 1990). Furthermore, the clearance of asparaginase is reduced by 50–80% in LDV-infected mice (Riley *et al.*, 1970; Nakayama *et al.*, 1990), indicating that LDV-permissive macrophages participate in the clearance of this enzyme.

Whether an increased release of enzymes from tissues in LDV-infected mice contributes to plasma enzyme elevations has not been resolved. This possibility has been considered in the case of SJL/J mice, because LDV infection causes an almost twofold higher plasma LDH elevation in these mice than observed in other mouse strains (Brinton and Plagemann, 1983). Because this difference was not related to a genetic difference in the rate of LDH-V clearance between SJL/J and Swiss mice, the results suggest that the higher LDH levels in infected SJL/J mice may reflect a higher influx of LDH into the circulation in these than in other strains of mice.

d. LDV Macrophage Receptor. The nature and location of the nonpermissive precursor cells that give rise to LDV permissive macrophages have not been elucidated. Neither have the nature and other properties of the subpopulation of LDV-permissive macrophages been defined. However, restriction of LDV replication to this subpopulation is probably mediated at the level of a surface component that acts as a receptor (Inada and Mims, 1984, 1985a; Kowalchyk and Plagemann, 1985; Buxton *et al.*, 1988). An incubation of cultured macrophages with trypsin renders the cells resistant to LDV infection, but after 12 hr of cultivation in the absence of trypsin they again become permissive (Kowalchyk and Plagemann, 1985). The LDV receptor has not been identified. One group has reported that LDV permissiveness strictly correlates with the surface expression of class II Ia antigens on macrophages (Inada and Mims, 1984, 1985a, 1987), but another group reported that the presence of I-A antigen does not correlate with LDV permissiveness (Kowalchyk and Plagemann, 1985; Buxton *et al.*, 1988; Onyekaba *et al.*, 1989b). The latter investigators found that although some productively infected macrophages were I-A antigen positive, the majority were not and many I-A-positive macrophages did not become infected. Furthermore, though up to 80% of the macrophages cultured from 1- to 2-week-old mice became LDV infected, only a few cells in these cultures expressed I-A antigen at a detectable level. In addition, LDV permissiveness of peritoneal macrophages from both newborn and adult mice was retained undiminished for at least 5 days during culture in medium containing colony-stimulating factor-1 (CSF-1), whereas the expression of I-A antigen was lost within 1 day (Buxton *et al.*, 1988; Onyekaba *et al.*, 1989b).

LDV replication has been observed in lines of macrophages from 1- to 2-week-old mice that became spontaneously transformed in culture, but maximally only 1–4% of the total cells in cultures of these lines became productively infected (Onyekaba *et al.*, 1989b). The permissive cells were frequently observed in small clusters. LDV-permissive cells were also present in cultures of 15/17 clones selected from one of the lines, but they represented only 0.5–1% of the total cells. These results suggest that all cells of these lines possess the potential of supporting LDV replication but that LDV permissiveness, presumably at the level of the LDV receptor, is only expressed transiently at a specific stage of differentiation, of growth or of the cell cycle. Regardless of the nature of the LDV receptor, electron microscopic evidence suggests that infection of macrophages by LDV occurs via receptor-mediated endocytosis (Kowalchyk and Plagemann, 1985).

It has been reported that upon infection of cultured macrophages

with LDV and 7 hr later with Sindbis virus (SV), a small proportion of the total virions formed (~1%) are pseudotypes with SV RNA and an LDV envelope, and that these pseudotype virions could infect chick embryo fibroblasts (Lagwinska *et al.*, 1975). These pseudotype virions were recognized by not being neutralized by anti-SV antibodies. The results have been interpreted to indicate that the limited host range of LDV is not due to a lack of suitable LDV receptors on nonpermissive cells but rather to an internal block in LDV replication (Lagwinska *et al.*, 1975; Rowson and Mahy, 1985). However, these results are subject to alternate interpretations. For example, this type of pseudovirion, besides expressing a high density of LDV glycoprotein, could have possessed low levels of SV attachment proteins sufficient for infection of chicken cells but not for neutralization by anti-SV antibodies. The formation of such pseudovirions seems quite possible because about 40% of the progeny were pseudotype virions possessing sufficient LDV and SV proteins to be neutralized by antibodies to either virus.

One intriguing suggestion concerning the nature of the LDV receptor on macrophages arises from the demonstration of a specific receptor on rat macrophages for LDH-V and other enzymes. It is likely that an analogous receptor mediates the clearance of LDH-V and probably of other enzymes by mouse macrophages. The possibility arises that this enzyme receptor may be restricted to LDV-permissive macrophages and may also function as the LDV receptor on these macrophages.

e. Electron Microscopy of Infected Macrophages. Electron microscopic and fluorescent antibody staining studies have shown that the replication of LDV in macrophages occurs in localized perinuclear areas (Stueckemann *et al.*, 1982a; Harty *et al.*, 1987a). Viral cores are formed abundantly in these areas (Fig. 2A) and mature by budding into single membrane cisternae (Brinton-Darnell *et al.*, 1975; Stueckemann *et al.*, 1982a) (Fig. 2B). The mode of virus release from the cells has not been elucidated but could occur via movement of virus-containing vesicles to the plasma membrane followed by exocytosis or by the disintegration of infected macrophages. No budding of LDV virions from the plasma membrane has been observed (Stueckemann *et al.*, 1982a). LDV infection of macrophages is associated with the rapid formation of large numbers of double-membrane vesicles, 100–300 nm in diameter (Stueckemann *et al.*, 1982a) (Fig. 2). These double-membrane vesicles have also been seen in mouse macrophages infected with MHV (Stueckemann *et al.*, 1982a). They do not seem to play a role in virus replication. They resemble to some extent autophagosomes, which have been studied most extensively in liver (Dunn, 1990a,b). These autophagocytic vesicles lack phosphatase activity and seem to arise from ribosome-

free smooth endoplasmic reticulum (Dunn, 1990a,b) by an undefined mechanism. Their formation is greatly enhanced in the liver by adverse conditions, such as amino acid starvation. Thus, if the double-membrane vesicles formed in macrophages infected with cytocidal viruses are autophagosomes, their formation could be triggered by localized stress imposed by the production of large amounts of virus material or by the production of specific viral products.

In macrophages that had been cultured for several weeks, the formation of large numbers of aberrant, elongated LDV virions has been observed (Stueckemann *et al.*, 1982a). These had the same diameter as spherical virions but reached lengths of up to 1 μm or more. Bacilliform virions have also been observed in alphavirus- and rubivirus-infected cells, but these were generally much shorter (Horzinek, 1981). The reason(s) for the formation of such aberrant virion particles is not known. It could reflect infection of a cell by a defective virus variant or result from a host function.

2. *Anti-LDV Immune Responses*

a. Polyclonal anti-LDV Responses during Natural Infection. Infection of mice with LDV results in a rapid antiviral antibody response (Cafruny and Plagemann, 1982a; McDonald *et al.*, 1983; Cafruny *et al.*, 1986a; Coutelier *et al.*, 1986). Low levels of anti-LDV IgM are only found early postinfection whereas anti-LDV IgG levels increase until about 2 months postinfection when they stabilize (see Fig. 1). Anti-LDV antibody formation is T cell dependent. Little or no anti-LDV antibodies are produced in nu^+/nu^+ BALB/c, AKR, and Swiss mice (Coutelier *et al.*, 1986; Onyekaba *et al.*, 1989a; Li *et al.*, 1990), and their formation is strongly inhibited by depleting mice of T cells by treatment with anti-T cell monoclonal antibodies (MAbs), especially depletion of $CD4^+$ T cells (Onyekaba *et al.*, 1989a; Li *et al.*, 1990). Most anti-LDV antibodies are directed to the glycoprotein VP-3, and are Ig_{2a} and IgG_{2b} isotypes (McDonald *et al.*, 1983; Cafruny *et al.*, 1986a; Coutelier *et al.*, 1986; Li *et al.*, 1990). However, significant levels of anti-LDV IgG_1 and IgG_3 are also produced (Coutelier *et al.*, 1986; Li *et al.*, 1990; Hu *et al.*, 1991). The isotype composition of anti-LDV antibodies may vary with the mouse strain and time after LDV infection (Coutelier *et al.*, 1986; Hu *et al.*, 1991).

The antibodies formed initially in LDV-infected mice do not neutralize LDV infectivity as measured by incubating antibody–virus mixtures *in vitro* and titrating residual infectivity in mice (Rowson and Mahy, 1985; Cafruny *et al.*, 1986a). Antibodies that neutralize LDV infectivity under these conditions begin to appear only 1–2 months

postinfection. Neutralization of LDV by antibodies in this assay is relatively inefficient and slow. LDV infectivity is reduced only about two orders of magnitude by incubation with undiluted immune plasma from persistently infected mice at 37°C for 1–2 hr and no neutralization is observed when the immune plasma is diluted 10–100 fold (Cafruny and Plagemann, 1982b; Cafruny *et al.*, 1986a; Harty and Plagemann, 1988). *In vitro* neutralization of LDV infectivity by either polyclonal or monoclonal anti-LDV antibodies correlates with an increase in the sedimentation rate of the virus from 230 S to ≥270 S (Plagemann *et al.*, 1991). Combined, the results suggest that the binding of multiple anti-LDV antibodies is required for neutralization of viral infectivity but the exact mechanism of neutralization is not known. Some anti-LDV antibody complexes formed *in vitro* or *in vivo* retain infectivity for mice that can be partly neutralized by incubation with antimouse IgG (sensitized virus; Notkins *et al.*, 1968; Cafruny *et al.*, 1986a; Coutelier and van Snick, 1988). Complement, on the other hand, has no effect on LDV infectivity, either in combination with neutralizing or nonneutralizing polyclonal or monoclonal anti-LDV antibodies (Rowson and Mahy, 1985; Cafruny *et al.*, 1986a; Harty and Plagemann, 1988). Whether infectious anti-LDV antibody–LDV complexes formed *in vitro* or *in vivo* infect mice by infecting macrophages via LDV or Fc surface receptors is not know. It has been demonstrated that anti-LDV antibodies from persistently infected mice enhance LDV infection of macrophages *in vitro* (Cafruny and Plagemann, 1982a; Inada and Mims, 1985a). *In vitro* immune enhancement is mediated via Fc receptors, but it is unclear whether the antibodies enhance the infection of only permissive cells or mediate the infection of normally nonpermissive cells. It seems doubtful that immune enhancement plays a role in persistent LDV infections of mice because immune suppression of persistently infected mice has no significant effect on LDV viremia (see below).

In general, neutralizing antibodies probably do not play a significant role in controlling LDV replication *in vivo* (see below). However, during the stage of persistent infection, practically all LDV is present in the circulation in infectious antibody–virus complexes (Notkins *et al.*, 1966a; Cafruny and Plagemann, 1982a; Hu *et al.*, 1991). These LDV–antibody complexes in plasma have been measured by binding of LDV infectivity to protein A or protein G and start to appear in the circulation at 1–2 weeks postinjection (Cafruny and Plagemann, 1982a; Hu *et al.*, 1991). These complexes seem equivalent to the anti-LDV antibody–LDV complexes formed *in vitro* in which LDV infectivity is sensitized to neutralization by antimouse IgG (Cafruny *et al.*, 1986b). Anti-LDV antibody–virion complexes appear to accumulate to some

extent in the kidney but do not cause immune complex disease (Porter and Porter, 1971; Oldstone and Dixon, 1971; Oldstone *et al.*, 1980), perhaps because they do not fix complement (Rowson and Mahy, 1985).

b. Anti-LDV Monoclonal Antibodies. Batteries of MAbs have been raised to native LDV and LDV that was inactivated by UV irradiation, glutaraldehyde treatment, or formalin treatment (Coutelier and van Snick, 1985; Coutelier *et al.*, 1986; Harty *et al.*, 1987a; Harty and Plagemann, 1988). In the course of these studies it was observed that spleen cells from acutely LDV-infected mice fuse with myeloma cells at least 10 times more efficiently than do spleen cells from uninfected mice (Coutelier and van Snick, 1985; Harty *et al.*, 1987a). This enhanced fusion activity of the spleen cells is probably related to the polyclonal activation of B cells caused by LDV infection (see later). The latter is also probably responsible for the low frequency of hybridomas producing anti-LDV MAbs observed in fusions of spleen cells from LDV persistently infected mice with myeloma cells (Coutelier and van Snick, 1985; Harty *et al.*, 1987a). Only 10 out of a total of 2846 hybridomas isolated from such fusions in two independent studies produced anti-LDV MAbs (Coutelier and van Snick, 1985; Harty *et al.*, 1987a). In another study (Coutelier *et al.*, 1986), a total of 22 anti-LDV MAbs has been reported to be derived from fusions of spleen cells obtained from mice at varying times postinfection (12 days to 14 months), but the overall efficiency of recovery of LDV-specific hybridomas was not stated, except that it was low. In contrast, when mice were first immunized with inactivated LDV and then infected with LDV a few days before removal of the spleen cells for fusion, up to 40% of the hybridomas isolated produced anti-LDV antibodies (Harty *et al.*, 1987a; Harty and Plagemann, 1988).

All anti-LDV MAbs that have been raised to LDV, whether native or inactivated, are directed to the glycoprotein of LDV, VP-3 (Coutelier *et al.*, 1986; Harty *et al.*, 1987a; Harty and Plagemann, 1988). One MAb has been raised to the nucleocapsid protein of LDV, VP-1, by immunization of mice with isolated nucleocapsids (Coutelier *et al.*, 1986). Anti-LDV MAbs have been of the IgM isotype and all four IgG isotypes, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3, probably reflecting the diversity of the normal anti-LDV antibody response of mice (see above). Some MAbs derived from *in vitro* fusions of spleen cells from persistently infected mice, as well as MAbs raised by immunization with UV-inactivated LDV, formalin-inactivated LDV, or LDV in Freund's complete adjuvant, neutralize LDV infectivity *in vitro* (Harty and Plagemann, 1988; Coutelier and van Snick, 1988).

Information on epitope specificity is available only for MAbs to formalin-inactivated LDV (Harty and Plagemann, 1988). All five neu-

tralizing MAbs to formalin-inactivated LDV that have been isolated interact with a single epitope of VP-3, but this epitope is not recognized during a natural infection of mice with LDV. The epitope is probably rendered immunogenic due to conformational changes of VP-3 caused by the formalin treatment. Nevertheless, the epitope must be expressed on native VP-3 because the MAbs neutralize LDV infectivity. Six non-neutralizing MAbs raised to formalin-inactivated LDV recognize three additional epitopes on VP-3. None of 12 anti-LDV MAbs raised to glutaraldehyde-inactivated LDV neutralizes LDV infectivity *in vitro* (Harty *et al.*, 1987a).

c. Failure of anti-LDV Immune Response to Control LDV Replication in Mice. Two perhaps related questions arise in relation to the effectiveness of the host anti-LDV response in controlling LDV infections; (1) Does it affect LDV replication in macrophages and (2) does it modulate LDV viremia? The answer to the first question is no. Passive immunization with immune plasma from persistently infected mice exhibiting neutralizing activity, with any of the neutralizing or non-neutralizing MAbs or with neutralizing rabbit anti-LDV antibodies (Cafruny and Plagemann, 1982b), does not significantly protect mice against LDV infection, even when very high concentrations of anti-LDV are administered (Cafruny *et al.*, 1986a; Harty and Plagemann, 1988). LDV infects and replicates equally well in passively immunized and in untreated mice. The following results further support the view that LDV replication in mice is impervious to all host defense mechanisms and also indicate that under most circumstances the latter have little effect on LDV viremia. First, the appearance of circulating neutralizing antibodies at 1–2 months postinfection has no significant affect on LDV viremia (Cafruny *et al.*, 1986a; Onyekaba *et al.*, 1989a). Second, LDV viremia is not affected by an inhibition of anti-LDV immune responses by treatment of mice with cyclosporin or depletion of T lymphocytes by anti-T cell MAb treatment or by splenectomy or lethal X-irradiation (Onyekaba *et al.*, 1989a). Third, LDV replication and long-term viremia are about the same in nude mice that fail to mount an anti-LDV immune response as in wild-type mice (Onyekaba *et al.*, 1989a). The plasma LDV levels of infected mice become continuously increased by about 1 log unit during weekly treatments with cyclophosphamide or dexamethasone over levels observed in untreated mice (DuBuy *et al.*, 1971; Onyekaba *et al.*, 1989a). Indirect evidence suggests that this increased viremia is not a consequence of the inhibition of the anti-LDV response, but more likely results from an increased formation of permissive macrophages and thus increased LDV replication in these mice (Onyekaba *et al.*, 1989a). This view is supported by the recent finding that a 1-day treatment of adult BALB/c mice with

200 mg cyclophosphamide/kg or 100 mg dexamethasone/kg increased the proportion of LDV-permissive macrophages in the peritoneum from 4.2 ± 0.2% to 18.8 ± 0.9 and 7.7 ± 0.3%, respectively (B. Hu and P. G. W. Plagemann 1990, unpublished observations). The persistent plasma LDV level of infected severe combined immune deficient (SCID) mice, which lack functional T and B lymphocytes, has also been found to be 1–2 log units higher than that of infected wild-type mice (Bradley *et al.*, 1991). The reason for the elevated LDV viremia in SCID mice has not been resolved. It contrasts with the normal LDV viremia in nude mice, which also fail to generate detectable levels of anti-LDV antibodies (Onyekaba *et al.*, 1989a). The proportion of LDV-permissive peritoneal macrophages in SCID mice seems to be much higher than in wild-type mice (Bradley *et al.*, 1991). Thus the elevated viremia could be due to an increased generation of LDV-permissive macrophages resulting in higher persistent LDV replication in these mice.

Overall, the results indicate that LDV has evolved properties that allow it to evade various known host defense mechanisms (Whitton and Oldstone, 1990). This includes cytotoxic T lymphocytes (CTLs), which play an important role in the control of infections by many animal viruses (Oldstone, 1989; Whitton and Oldstone, 1990). It is unclear at present whether no LDV-specific CTLs are generated in LDV-infected mice or whether LDV-infected macrophages are resistant to attack by such cells. Because LDV matures by budding into intracytoplasmic cisternae, it is possible that intact LDV proteins are not expressed on the surface of LDV-infected macrophages and the latter might be resistant to antibody-dependent, cell-mediated cytotoxicity (ADCC). However, infected macrophages should express peptides of LDV proteins in context of the class I MHC antigen on their surface and thus be recognized by CTLs.

Interferon (IFN) or the development of defective interfering particles or temperature-sensitive mutants of LDV also seem not to play a role in the persistent LDV infection of mice (Stueckemann *et al.*, 1982a,b). IFN-α/β is only transiently detected in infected mice 1-day postinfection (Rowson and Mahy, 1985). It is probably produced by the large number of LDV-permissive macrophages that become productively infected during the primary infection of a mouse (Lussenhop *et al.*, 1982). Macrophages that become infected during the subsequent persistent phase of infection probably also produce IFN-α/β, but since their numbers are relatively small, the plasma concentration of IFN-α/β remains below detectable levels. LDV replication in mouse macrophages is relatively resistant to IFN-α/β as compared to MHV replication in these cells (Stueckemann *et al.*, 1982a). Futhermore, freshly explanted mouse

peritoneal macrophages generally exhibit resistance to productive infection by vesicular stomatitis and encephalomyocarditis (EMC) viruses due to the presence of low concentrations of endogenous IFN-α/β (Belardelli *et al.*, 1984), but seem to become susceptible upon *in vitro* culture. This is not the case for LDV; macrophages are equally susceptible to LDV infection whether infected immediately after harvest or after several days in culture (Stueckemann *et al.*, 1982a).

It is still unclear what properties of LDV allow it to escape all host defense mechanisms. Its mode of persistence seems to differ from the persistent infections established by various other viruses, which require the development of virus variants and modulation or down-regulation of the host immune response to them or both (Oldstone, 1989; Whitton and Oldstone, 1990). In common with many persistent viruses, however, LDV replicates in macrophages that normally play a role in the defense against virus infections.

3. *Effect of LDV Infection on Host Immune Responses*

a. Cellular Immune Responses. The acute phase of LDV infection is associated with a depression of cellular immune responses of mice as generally measured by an increased survival of tissue or tumor transplants, but this effect is only transient (Howard *et al.*, 1969). These findings have been previously summarized (Rowson and Mahy, 1975, 1985; Cafruny, 1989).

LDV infection also results in reduced delayed-type hypersensitivity and lipopolysaccharide (LPS)-induced inflammatory responses. IFN-γ (Heremans *et al.*, 1987) and the formation of T suppressor cells (Inada and Mims, 1986a) have been implicated in this effect.

In addition, experimental allergic encephalitis (EAE) induced in mice by injection of myelin basic protein (MBP) is greatly reduced in LDV-infected mice as compared to uninfected mice (Inada and Mims, 1986b; Billiau *et al.*, 1988). This effect was attributed in one study to a destruction of I-A-positive, antigen-presenting macrophages by LDV infection (Inada and Mims, 1986b) and in the other study to the formation of IFN-γ in LDV-infected mice, because injection of IFN-γ or IFN-α/β had a similar effect, whereas the treatment of mice with anti-IFN-γ antibodies increased the incidence of EAE (Billiau *et al.*, 1988).

b. Polyclonal Activation of B Cells and Immune Complex Formation. In contrast to the suppressed cellular immune responses during the primary infection, humoral antibody responses, both T cell dependent and independent, are generally enhanced during this early phase of infection (Rowson and Mahy, 1975, 1985; Cafruny, 1989). The effect is

probably causally related to initiation of the polyclonal activation of B cells rapidly caused by LDV infection (Notkins *et al.*, 1966b; Cafruny and Plagemann, 1982a; McDonald *et al.*, 1983; Michaelides and Simms, 1977; Coutelier and van Snick, 1985; Li *et al.*, 1990; Coutelier *et al.*, 1990). During the chronic phase of the infection, on the other hand, the humoral anitbody response, at least to T cell-dependent antigens, is generally somewhat reduced (Rowson and Mahy, 1985; Michaelidis and Simms, 1980). LDV infection also inhibits the formation of autoantibodies to nuclear antigen and DNA in New Zealand mice (Oldstone and Dixon, 1972).

A number of observations are explained by or reflect the initiation of the polyclonal activation of B cells by LDV infection and its persistence: (1) the increased fusion activity of B cells from LDV-infected mice mentioned already, which is transient; it peaks at about 5 days postinfection and then decreases progressively to normal levels by 4–5 weeks postinfection (Coutelier and van Snick, 1985); (2) an increased activation of B or T cells or both in LDV-infected mice indicated by an increased spontaneous [^{3}H]thymidine incorporation of spleen cells from these mice *in vivo* as well as *in vitro* (Coutelier and van Snick, 1985; Li *et al.*, 1990; Coutelier *et al.*, 1990); (3) an increased *in vitro* production of IgG_{2a} by spleen cells from 3- to 5-day infected mice (Coutelier and van Snick, 1985; Coutelier *et al.*, 1990) and an increased number of spleen cells that spontaneously produce antibodies to sheep red blood cells and dinitrophenol (Michaelides and Simms, 1980); (4) a reduction in the response of spleen cells from infected mice to concanavalin A (Con A) and LPS (Li *et al.*, 1990); (5) an increase in spleen mass (Rowson and Mahy, 1985; Li *et al.*, 1990) and of germinal centers in the spleen (Proffitt *et al.*, 1972); and (6) an increase in the levels of Ig in the plasma of infected mice. The latter have been quantitated as a function of time after infection in a number of mouse strains using isotype-specific capture enzyme-linked immunosorbent assays (ELISAs) (Li *et al.*, 1990; Hovinen *et al.*, 1990) or a radioimmune assay (Coutelier and van Snick, 1985). Plasma Ig levels begin to increase within a few days postinfection and reach maximum levels 4–6 weeks postinfection, coincidental with the formation of anti-LDV antibodies (see Fig. 1). The polyclonal activation is generally IgG_{2a}-specific, although in some mice IgG_{2b} rather than IgG_{2a} increases have been observed (Li *et al.*, 1990). Levels of IgG_{2a} or IgG_{2b} increase from 0.01 to 0.5 mg/ml in uninfected mice maximally to 4 to 16 mg/ml in infected mice depending on the mouse strain (Coutelier and van Snick, 1985; Li *et al.*, 1990) (Fig. 1). Plasma levels of IgG_1 and IgG_3 are generally not elevated. When in-

creases occur they are minor and may not be related to LDV infection (Li *et al.*, 1990). Plasma IgM levels may become elevated slightly but only transiently during the first 2 weeks postinfection.

A polyclonal activation of B cells has been associated with other viral infections (Ahmed and Oldstone, 1984), but the effect of LDV infection is quite unique in magnitude and persistence of the effect. A similar polyclonal activation of B cells, however, has been observed in mice infected with a defective retrovirus that causes AIDS-like symptoms in mice (MAIDS) (Pattengale *et al.*, 1982; Mosier *et al.*, 1985; Huang *et al.*, 1989; Chattopadhyay *et al.*, 1989), but in this case, besides increases in plasma IgG_{2a}, the levels of IgG_{2b} and IgG_1 are also generally elevated (Pattengale *et al.*, 1982; Mosier *et al.*, 1985; Aziz *et al.*, 1989; Even *et al.*, 1991). A similar polyclonal activation of B cells is also associated with AIDS in humans (Fauci, 1988) and may be a general feature of persistent viral infections.

The mechanism of polyclonal activation of B cells by LDV infection has not been resolved, but a number of observations are pertinent in this respect: (1) The polyclonal activation of B cells is inhibited by treatment of infected mice with cyclophosphamide, which is highly toxic to proliferating lymphoidal cells (Bach and Strom, 1985), but does not inhibit LDV replication in macrophages (Li *et al.*, 1990). (2) It is largely dependent on functional T cells. Only small increases in plasma IgG_{2a} or IgG_{2b} are observed in nude mice or in mice that are depleted of $CD4^+$ T helper (Th) cells by treatment with anti-T cell MAbs (Li *et al.*, 1990; Coutelier and van Snick, 1985; Coutelier *et al.*, 1990). (3) B lymphocytes isolated from 7-day LDV-infected mice exhibit some spontaneous proliferation and IgG_{2a} production when cultured *in vitro*. In contrast, B cells isolated from LDV-infected, T cell-depleted mice, though they are activated as measured by [^{3}H]thymidine incorporation *in vitro*, fail to produce IgG_{2a} (Coutelier *et al.*, 1990). The latter cells, however, can be induced to produce IgG_{2a} by incubation with IFN-γ (Coutelier *et al.*, 1990). (4) A related finding is that spleen cells from 5- to 7-day LDV-infected mice on *in vitro* culture produce significant amounts of IFN-γ, which is not observed in cultures of spleen cells from uninfected mice (P. G. W. Plagemann *et al.*, unpublished observations). Furthermore, spleen cells from LDV-infected mice produce IFN-γ more rapidly and in higher amounts when cultured with ConA than do spleen cells from uninfected mice. (5) The polyclonal activation of B cells is observed in actively infected mice, but not in mice immunized with inactivated LDV (Cafruny *et al.*, 1986a), and thus the productive infection of macrophages must play a critical role. Combined, these observa-

tions support the following speculative model (Li *et al.*, 1990; Coutelier *et al.*, 1990), which may be applicable to the polyclonal B cell activation mediated by other viruses.

The productive, cytocidal replication of LDV in a large number of permissive macrophages during a primary infection of a mouse is probably associated with the release of numerous lymphokines. For example, considerable levels of IFN-α/β are transiently produced during the first 2 days postinfection (Rowson and Mahy, 1985), which seems responsible for the activation of the remaining nonpermissive macrophages as well as natural killer cells observed during this phase of the infection (Stevenson *et al.*, 1980; Lussenhop *et al.*, 1982; Koi et al., 1981). In addition, it seems likely that both interleukin 1 (IL-1) and IL-6, which function as B lymphocyte growth or differentiation factors (Kishimoto and Hirano, 1988; Vink *et al.*, 1988), are produced by LDV-infected or activated macrophages. It has been shown that IL-6 in combination with IL-1, but not alone, induces the proliferation of and IgM production by resting murine lymphocytes (Vink *et al.*, 1988).

Thus, one model suggests that B cell activation in LDV-infected mice may be triggered by IL-1 and IL-6, perhaps in combination with an LDV protein (or proteins) acting as a polyclonal B cell activator (Coutelier *et al.*, 1990) comparable to dextran sulfate and LPS (DeFranco, 1987). The differentiation of the activated B cells to IgG-producing plasma cells is then thought to be mediated by cytokines produced by T cells that become activated in the course of the anti-LDV immune response. It is postulated that LDV antigens may be preferentially presented to and activate Th cells of subtype 1 to explain the specific elevation of plasma IgG_{2a} in LDV-infected mice. Th 1 cells produce IFN-γ and IL-2 (Mossmann and Coffman, 1989; Paul, 1989) and IFN-γ is known to induce (or switch to) the formation of IgG_{2a} by activated B cells, whereas IL-4 produced by Th cells of subtype 2 enhances the production of IgG_1 and IgG_3 by activated B cells (Snapper and Paul, 1987; Snapper *et al.*, 1988; Finkelman *et al.*, 1988; Mossmann and Coffman, 1989). In addition, productively infected macrophages are expected to present LDV antigens in context of the class I antigens to $CD8^+$ cells, which are also known to produce IFN-γ (Mossmann and Coffman, 1989). One unexplained observation is the formation of IgG_{2b}- rather than IgG_{2a}-producing B cells in some LDV-infected mice (Li *et al.*, 1990). This finding is surprising because the production of IgG_{2a} and IgG_{2b} is thought to be differently regulated (Mossmann and Coffman, 1990).

The second model suggests that one of the LDV proteins may function as a superantigen that activates a whole subset of Th cells by directly interacting with the Vβ subunit of their T cell receptor and a nonpo-

lymorphic segment of the class II MHC antigen of B cells, similarly as has been demonstrated for mouse mammary tumor virus-related Mls antigens and various bacterial toxins (Marrack and Kappler, 1990). Such nonspecific cognate interaction between Th and B cells results in the polyclonal activation of the B cells and their differentiation into IgG-producing plasma cells, which is driven by cytokines produced by the superantigen-activated T cells (Tumang *et al.*, 1991). A precedent for the second model is the finding that the single gene product of the defective MAIDS murine leukemia virus (MuLV) functions as superantigen (Cerny *et al.*, 1990; Hügin *et al.*, 1991). The latter hypothesis could also explain the general stimulation of humoral antibody responses in acutely LDV-infected mice because the interaction between B and T cells mediated by superantigen has been shown also to provide selective help for the proliferation of B cells that have encountered a specific antigen (Tumang *et al.*, 1991). In addition, a specific activation of Th1 and $CD8^+$ cells envisioned in either model could explain the finding that antiviral antibodies generated during virus infections of mice are also predominantly IgG_{2a}, whereas those generated to administered soluble antigens are primarily IgG_1 (Coutelier *et al.*, 1987).

INF-γ is also known to enhance the expression of I-A surface antigen on macrophages (Mengle-Gaw and McDevitt, 1985; Unanue and Allen, 1987). In LDV-infected mice, the expression of I-A antigen by peritoneal macrophages not killed by LDV infection and their antigen-presenting activity decrease drastically during the first day postinfection (Isakov *et al.*, 1982a,b,c; Inada and Mims, 1985a; Buxton *et al.*, 1988), in spite of the fact that many of these macrophages become activated as measured by an increased expression of Fc and complement receptors (Lussenhop *et al.*, 1982). The loss of I-A antigen, however, is only transient; I-A antigen reappears on macrophages 3–7 days postinfection. Its reexpression could be related to the production of IFN-γ at this time, because it is enhanced by i.p. injection of starch and ConA (Buxton *et al.*, 1988). The reason for the initial loss of I-A antigen on macrophages after LDV infection is not clear. In part, it might be due to cytocidal infection by LDV, but it is probably primarily mediated directly or indirectly by cytokines produced by the infected macrophages. Two effects can be envisioned. On the one hand, prostaglandins produced by macrophages are known to suppress I-A antigen expression (Mengle-Gaw and McDevitt, 1985; Unanue and Allen, 1987). On the other hand, IL-1 produced by macrophages induces the release of corticosterone by the adrenal cortex (Durum *et al.*, 1985), which in turn is known to suppress macrophage and T cell functions, including the expression of I-A antigen on macrophages (Kelso and Munck, 1984).

The latter suggestion is supported by the finding of a transient elevation of plasma corticosterone concentrations in mice after LDV infection (Riley, 1981; I. K. Buxton and P. G. W. Plagemann, unpublished observations). Corticosterone release could also be responsible for the suppression of cellular immune responses during the acute phase of infection with LDV (see Section II,3,a) and the reduced responsiveness of spleen cells to ConA during the acute phase of infection.

Besides infectious virion-antibody complexes, immune complexes of 150–300 S are formed in LDV-infected mice (Cafruny *et al.*, 1986b). These are recognized and quantified by their binding in the presence of Tween 20 to high-affinity protein-binding ELISA plates without prior coating of the plates with any antigen (Cafruny *et al.*, 1986a,b). Their formation correlates with the polyclonal activation of B cells as reflected by increases in plasma IgG_{2a} levels and with the production of anti-LDV antibodies (Fig. 1). These complexes are unstable at pH 3 and contain immunoglobulin, but the potential antigens present in these complexes have not been identified (Cafruny *et al.*, 1986b; Hu *et al.*, 1991). Their formation is probably a consequence of the polyclonal activation of B cells in LDV-infected mice, rather than being related to the formation of anti-LDV antibodies, because the immunoglobulin isotypes associated with these immune complexes correlate with the isotype specificity of the polyclonal activation of B cells, either IgG_{2a} or IgG_{2b}, whereas the anti-LDV antibodies exhibit a much broader isotype specificity (Hu *et al.*, 1991; Even *et al.*, 1991). One possibility is that these complexes are composed of cellular antigens and autoantibodies formed in the course of the polyclonal activation of B cells. Autoantibodies to Golgi vesicles (Weiland *et al.*, 1987, 1990) and to undefined brain, liver, kidney, and spleen-derived antigens (Cafruny and Hovinen, 1988a), as well as antibodies to a surface antigen of MuLV-transformed mouse cells (Weiland *et al.*, 1990), have been demonstrated in LDV-infected mice. A formation of antibodies to autoantigens is also associated with the polyclonal activation of B cells in MAIDS MuLV-infected mice (Klineman and Steinberg, 1987) and the formation of immune complexes in these mice (Even *et al.*, 1991).

Plasma from LDV-infected mice has also been examined for immune complexes that bind C1q. In one study, none was observed in SWR/J mice 4–10 weeks postinfection with LDV (Oldstone *et al.*, 1980). In contrast, another study reported the transient presence in the plasma of BALB/c mice of IgM and IgG_1-containing C1q-binding complexes between 3 and 9 days and between 6 and 12 days postinfection, respectively, whereas IgG_2-containing C1q-binding complexes began to appear about 15 days postinfection (McDonald *et al.*, 1983). These

complexes were thought to be composed of anti-LDV antibodies and virions, but instead they might represent the low-molecular-weight immune complexes that bind to ELISA plates and are formed in association with the polyclonal activation of B cells. This view is supported by a failure to detect LDV antigens in the C1q-binding complexes (McDonald *et al.*, 1983) and the findings that complement has no effect on the infectivity of anti-LDV–virion complexes and that LDV–antibody complexes arise later than the C1q-binding complexes (see Section II,A,2a).

4. Pathology

In spite of the continuous destruction of the LDV-permissive macrophage population during infection of mice with LDV and the permanent polyclonal activation of B cells and perhaps other immunological changes observed in these mice, no clinical symptoms are associated with LDV infections, except under the multifactorial conditions leading to paralytic disease (see Section II,B). However, transient histological CNS changes have been observed in 4- to 6-week-old C57BR/cdJ mice during the acute phase of infection with LDV. In these mice, encephalomyeloradiculitis inflammatory lesions in the white matter developed after peripheral inoculation of LDV whether or not the mice were immunosuppressed and regardless of age or sex (Stroop and Brinton, 1983). The lesions in C57BR/cdJ mice resembled those observed in early stages of EAE. Injection of these mice with spinal cord extracts in Freund's complete adjuvant 1 week before LDV infection caused a greater incidence and severity of the lesions than either LDV infection or immunization with spinal cord extracts alone (Stroop and Brinton, 1985). This effect seems opposite to the suppression of EAE by LDV infection in SJL/J and or C57BL/6 mice (Billieu *et al.*, 1988; Inada and Mims, 1986b). The CNS histopathology induced by LDV infection in C57BR/cdJ mice cannot involve an ecotropic provirus, as is implicated in the etiology of ADPM, because C57BR/cdJ mice carry only a single defective ecotropic provirus (emv-2) (Coffin, 1985) and C57BL/6 mice, which carry the same provirus, did not develop comparable CNS lesions after LDV infection (Stroop and Brinton, 1983). CNS histopathology was also not observed in C57L/J mice after LDV infection, except for a low incidence of transient mild encephalitis (Stroop and Brinton, 1983).

5. Synopsis

LDV invariably establishes a lifelong, asymptomatic, persistent infection in all strains of mice regardless of sex and age of infection. The persistent infection is associated with continuous viremia, a 5- to 10-

fold elevation of plasma LDH-V activity, and subtle effects on the host immune system. Especially prominent is a persistent polyclonal activation of B cells that is reflected by an IgG_{2a}- or IgG_{2b}-specific hypergammaglobulinemia. Productive LDV infection in the mouse is restricted to a subpopulation of macrophages with a surface component that acts as LDV receptor. Productive infection of macrophages results in the release of about 10^4 ID_{50}/cell and is highly cytocidal. The persistent infection in the mouse is maintained by a slow but continuous regeneration of LDV-permissive macrophages that become infected as soon as they arise. A normal function of the permissive macrophages seems to be the clearance of LDH-V and of some other enzymes from the circulation, and the continuous destruction of the permissive macrophages accounts for the elevation of these enzymes in the circulation. The polyclonal activation of B cells seems triggered by the release of cytokines and perhaps LDV proteins from the infected macrophages. The persistent infection is maintained in spite of a vigorous anti-LDV antibody response of the mouse, which is primarily directed to the viral envelope glycoprotein VP-3. The production of anti-LDV antibodies results in the sequestration of all LDV in infectious antibody–virion complexes in which infectivity is resistant to complement. LDV successfully escapes all host defense mechanisms. Passive immunization with polyclonal or monoclonal anti-LDV antibodies that neutralize LDV infectivity *in vitro* also does not significantly protect mice from LDV infection.

B. Age-Dependent Poliomyelitis

1. Multifactorial Etiology and Pathology

The motor neuron disease, ADPM, was first observed in old C58/M mice after transplantation of Ib tumor cells (Murphy *et al.*, 1970). The disease was first thought to be immunologically induced, but it was surprising that the incidence of the paralytic symptoms was increased by treatment of the mice with cyclophosphamide or X-irradiation, that is, by immunosuppressive treatments (Murphy *et al.*, 1970). Only later was it found that the disease in these mice was caused by LDV that contaminated the transplanted Ib tumor cells (Nawrocki *et al.*, 1980; Martinez *et al.*, 1980).

Paralytic symptoms occur generally between 1 and 4 weeks after peripheral inoculation of susceptible mice with a neurovirulent strain of LDV (see Section II,C-3). The latent period seems to decrease with an increase in age of the mice between 5 and 18 months of age (Stroop and

Brinton, 1983; Harty and Plagemann, 1990; see below). The paralytic disease is associated with neurophagia of spinal cord and brain stem motor neurons (Homburger *et al.*, 1973; Murhpy *et al.*, 1983; Stroop and Brinton, 1983; Stroop *et al.*, 1985). No brain involvement per se is detectable.

ADPM is caused by LDV infection only under a specific set of conditions. It exhibits certain similarities to amyotropic lateral sclerosis of humans (Rowland, 1982, 1991; Murphy *et al.*, 1983; Contag *et al.*, 1989, 1991) in that susceptibility of both diseases is age related and depends on genetic and environmental factors, and the pathology is largely limited to neurons. In the case of ADPM, destruction of anterior horn motor neurons has been shown to be the result of a cytocidal infection by LDV (Contag *et al.*, 1986a; Brinton *et al.*, 1986b; Contag and Plagemann, 1989; see Secion II,B,2), but the infection of motor neurons and the progression and spread of the infection of motor neurons through the spinal cord are dependent on two additional factors, respectively. First, the susceptibility of the motor neurons to LDV infection is dependent on the expression of an endogenous ecotropic MuLV in the anterior horn motor neurons (see Section II,B,3). Second, the spread of the cytocidal motor neuron infection ultimately resulting in paralysis is blocked by an anti-LDV immune response. Thus ADPM develops only when the immune response of susceptible mice to LDV is impaired either as a result of old age or environmental influences. Anti-LDV immune responses specifically protect the motor neurons from LDV infection without affecting the replication of the virus in permissive macrophages (see Section II,B,3).

Neuronal loss is generally, but not always, associated by mononuclear cell infiltration. For example, in dexamethasone-treated mice, motor neuron destruction occurred in the absence of a significant inflammatory response (Kascsak *et al.*, 1983) and LDV-infected very old C58 mice developed paralysis very rapidly without signs of inflammation in the spinal cord (Stroop *et al.*, 1985). Furthermore, LDV replication in neurons and their destruction seem generally to precede the influx of inflammatory cells (Brinton *et al.*, 1986b; Contag and Plagemann, 1989). Inflammation is therefore thought to be a consequence of cytocidal LDV infection of motor neurons rather than the cause of motor neuron destruction.

The proposed model of the interaction of the expression of endogenous ecotropic MuLV in motor neurons, motor neuron infection by LDV, and immunosuppression in the etiology of ADPM is illustrated in Fig. 3. It serves as the basis for discussing the experimental findings supporting this model in the following sections.

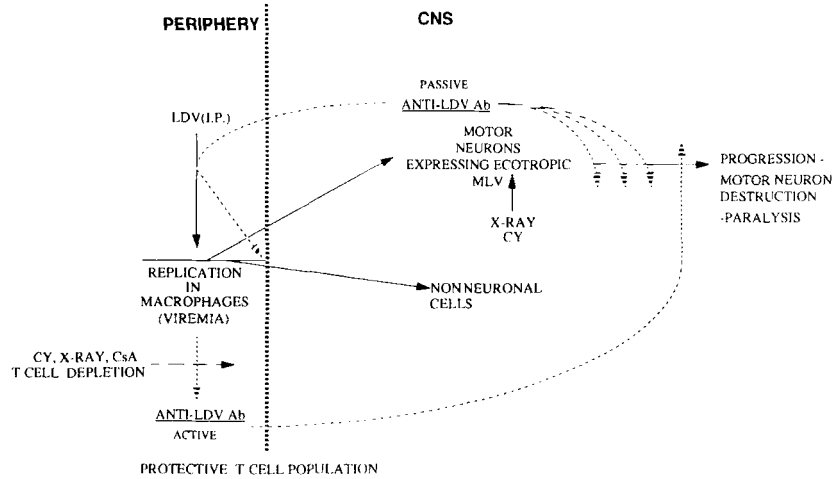

FIG. 3. Model for the interaction between the formation of ecotropic MuLV RNA in spinal cord motor neurons, suppression of the anti-LDV immune response, and cytocidal replication of a neurovirulent isolate of LDV in motor neurons in the etiology of ADPM; CY, cyclophosphamide; CsA, cyclosporin A. For the further explanations see text.

2. *Cytocidal Infection of Motor Neurons by LDV*

In situ hybridization studies have shown that motor neuron destruction leading to paralysis is invariably associated with the accumulation of LDV RNA in spinal cord motor neurons, specifically in areas of motor neuron destruction, that is, in the lumbar region of mice with hind limb paralysis or in the cervical region of mice with front limb paralysis (Contag *et al.*, 1986a; Contag and Plagemann, 1989) (Figs. 4B and 4C). The presence of LDV RNA in motor neurons increases progressively with time after LDV inoculation and this increase has been quantitated by Northern blot hybridization of spinal cord RNA (Contag and Plagemann, 1989; Contag *et al.*, 1989) (Fig. 4A). The results indicate that only a limited number of cells in the spinal cord become initially infected after a peripheral inoculation of LDV and that the cytocidal infection by LDV then spreads progressively between neurons until neuronal destruction is sufficient to result in paralytic symptoms. This time course of protracted infection of motor neurons in the spinal cord contrasts sharply with the rapid replication of LDV in macrophages in the periphery (Fig. 4A). As discussed already, high levels of LDV RNA and antigens are found only transiently in other tissues outside the central nervous system, such as the spleen, and the plasma during the

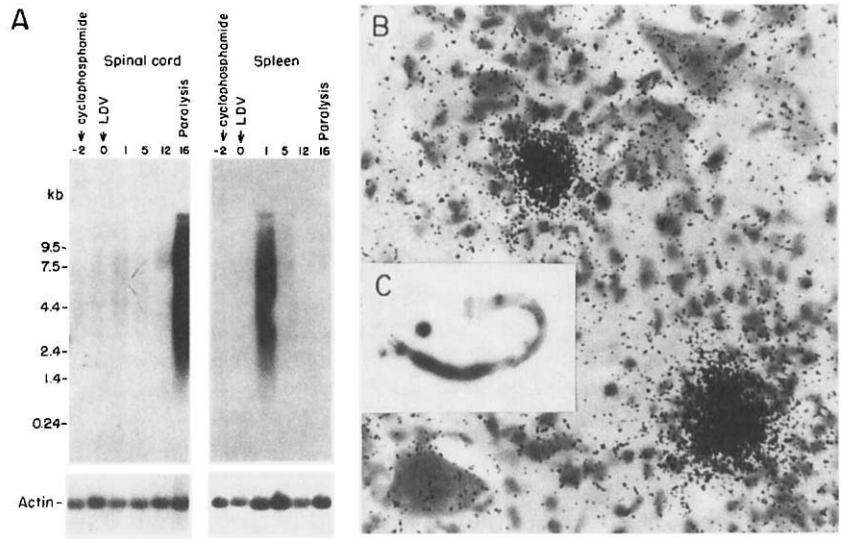

FIG. 4. Levels of LDV RNA in the spinal cords and spleens of immunosuppressed 6-month-old C58/M mice as function of time postinfection with LDV-M (A) and cellular location of LDV RNA in the spinal cord of a paralyzed mouse 16 days postinfection (B) and (C). A group of mice was injected with cyclophosphamide (200 mg/kg) and 1 day later infected with 10^6ID_{50} of LDV-M per mouse. RNA was extracted from the spinal cords and spleens of groups of three mice at the times indicated and analyzed by Northern blot hybridization using a ^{32}P-labeled, LDV-specific cDNA (4–11) probe and subsequently with a ^{32}P-labeled, actin-specific oligonucleotide probe (A). Sections of the spinal cord of a mouse with hind limb paralysis (at 16 days postinfection) were hybridized *in situ* with the LDV-specific cDNA probe labeled with ^{125}I (B and C). [Data from Contag and Plagemann (1989); Contag *et al.* (1989); and C. H. Contag and P. G. W. Plagemann unpublished observations (1989).] Magnification (in B): about ×400.

first 1–2 days postinjection, because all permissive macrophages present in a mouse at the time of infection are destroyed during the initial acute phase of infection. The levels of LDV RNA in the spinal cord at the time of paralysis are comparable to those found in the spleen about 1 day postinjection with LDV on the basis of percentage of total tissue RNA (Contag and Plagemann, 1989; Contag *et al.*, 1989) (Fig. 4A).

To what extent LDV infection of motor neurons results in the production of infectious virions has not been established. In one study (Kascsak *et al.*, 1983), the levels of infectious LDV in extracts of spinal cords of perfused C58 mice with signs of paralysis were several orders of

magnitude higher than those of extracts of spinal cords from nonparalyzed companion mice, and also 1–2 log units higher than the plasma LDV levels of these mice. On the other hand, in two other studies, conducted under comparable experimental conditions, the spinal cord LDV levels were about the same as those of plasma and thus could have reflected blood-borne virus (Stroop and Brinton, 1983; Cafruny *et al.*, 1986c). No LDV particles were detected by electron microscopy in sections of spinal cords of older immunosuppressed C58 mice at the time of paralysis (Stroop *et al.*, 1985), that is, at the time of maximum accumulation of LDV RNA in the spinal cord (Fig. 4). The protracted presence of LDV RNA in spinal cord motor neurons may suggest that LDV replication in motor neurons is slow and perhaps less efficient than in macrophages. However, other factors may contribute to the protracted LDV infection of motor neurons in C58 mice. For example, viral spread among cells in the spinal cord probably involves diffusion through the extracellular spaces of the neuropil, which might be greatly impeded by the very small size of this space (Johnson *et al.*, 1988). There is also the possibility that LDV spread between neurons is mediated via direct cell–cell interaction and that this process is inefficient. LDV RNA is also detected in smaller nonneuronal cells in the spinal cord, which are suspected to be microglial cells (Contag *et al.*, 1986a; Contag and Plagemann, 1989), but whether they have any function in motor neuron infection is unclear.

C58/M mice and AKR mice 1–5 months of age seldom develop paralytic disease after LDV infection, even when given a single injection of cyclophosphamide before LDV inoculation (Murphy *et al.*, 1980) (Table II), but 4- to 6-week-old C58/M mice, after receiving a single injection of cyclophosphamide before LDV infection, may develop chronic histological poliomyelitis that can persist for many weeks without obvious neurological dysfunction (Stroop and Brinton, 1983). LDV RNA, LDV antigens, and LDV particles have been detected by *in situ* hybridization, immunocytochemical staining, and electron microscopy, respectively, in spinal cord sections of these mice, presumably in motor neurons, but the number of cells infected seems to have been low (Stroop and Brinton, 1985; Brinton *et al.*, 1986b). LDV RNA has also been found in nonneuronal cells in these young C58/M mice (Brinton *et al.*, 1986b).

3. *Expression of Ecotropic Murine Leukemia Virus in Motor Neurons as Predisposing Factor for ADPM Susceptibility*

Genetic studies have shown that the susceptibility of mice to ADPM is linked to the presence of multiple proviral copies of N-tropic, ecotropic MuLV and homozygosity of the allele at the locus that permits the

TABLE II

EFFECT OF AGE AND SOURCE OF C58 AND AKR MICE AND OF MODE OF ADMINISTRATION OF CYCLOPHOSPHAMIDE ON INCIDENCE OF ADPM[a]

Mice	Age (months)	Paralyzed mice/total number of mice after cyclophosphamide treatment[b]		
		None	1 day before LDV	1 day before LDV + weekly thereafter
C58/M	1	—	—	7/13 (23 ± 5)
C58/M	2	—	—	3/3 (34 ± 3)
C58/M	3	0/6	1/11 (ND)	4/5 (34 ± 2)
C58/M	4–5	0/2	2/6 (23,31)	7/8 (23 ± 2)
C58/M	6–8	1/27 (ND)	130/140 (16 ± 0.2]	—
C58/M	10	2/12 (ND)	14/14 (15 ± 1]	—
C58/M	11–12	4/9 (32, ND)	15/16 (17 ± 2)	—
C58/M	14–20	7/11(14 ± 2)	—	—
C58/J	6–7	—	6/17 (20 ± 1)	2/2 (19, 25)
AKR/Boy	6–7	—	10/10 (16 ± 1]	—
AKR/NIH	6–7	—	0/3	—
AKR/J	6–7	—	0/65	7/8 (25 ± 2)
AKR/J *nu/nu*	2	2/2 (37, 39)	2/2 (18, 22)	—
AKR/J[c]	2–3	0/2	0/2	4/4 (27 ± 2)

[a] The mice were injected with 200 mg cyclophosphamide/kg as indicated and with 10^6 ID^{50} of LDV-v (day 0); they were observed for paralytic symptoms until 40–60 days postinfection (G. W. Anderson *et al.*, unpublished observations 1991).

[b] Numbers in parentheses represent onset of paralysis (days postinfection, mean ± SEM); ND, not determined.

[c] AKR $nu^+/+$ or wild type.

efficient replication of N-tropic MuLV (*Fv-1*$^{n/n}$) (Pease *et al.*, 1982; Pease and Murphy, 1980; Murphy *et al.*, 1983). This genetic background is found in C58, AKR, C3H/FgBoy, and PL/J mice. Nearly 100% of the mice of these strains develop paralytic disease after infection with a neurovirulent strain of LDV when at least 6 or 9 months of age and when X-irradiated (600 rads) 1 day before LDV infection (Pease and Murphy, 1980; Murphy *et al.*, 1983).

In AKR and C58 mice, ecotropic MuLV provirus expression becomes apparent shortly before or after birth, resulting in MuLV replication primarily in lymphoid tissues (Pincus, 1980; Kozak, 1985; Coffin, 1984, 1985). The development of T cell lymphomas in these highly leukemic mouse strains is a consequence of the formation of oncogenic recombinants between the replicating ecotropic MuLV and sequences of endog-

enous xenotropic and polytropic MuLV (Stoye and Coffin, 1987; Laigret *et al.*, 1988; Stoye *et al.*, 1991). In contrast, the susceptibility of old C58 and AKR mice to ADPM correlates with the expression of the endogenous ecotropic MuLV in spinal cord and brain stem motor neurons (Contag and Plagemann, 1988, 1989; Contag *et al.*, 1989). MuLV RNA in the spinal cord has been recognized by Northern blot hybridization analyses of total spinal cord RNA with an oligonucleotide probe specific for the *env* gene of ecotropic MuLV, and its cell distribution in the spinal cord was analyzed by *in situ* hybridization using a full-length ecotropic provirus (AKR-623) (Herr, 1984) as probe. The expression of the endogenous ecotropic MuLV in motor neurons seems to increase with age of the mice. Only low levels of ecotropic MuLV RNA were detected by Northern blot hybridization analyses in the spinal cords of 2- to 3-month-old C58/M mice, which show low susceptibility to ADPM, even when receiving a single immunosuppressive treatment 1 day before infection with neurovirulent LDV (Table II). Much higher levels of both full-length 8.2- and 3-kb ecotropic MuLV RNA (presumably MuLV *env* mRNA) were detected in the spinal cords of 6- to 7-month-old C58/M mice (see Fig. 5A), at which time the mice are highly susceptible to ADPM provided they are immunosuppressed (see Tables II and III). *In situ* hybridization has shown that most of the ecotropic MuLV RNA in the spinal cord is located in motor neurons throughout the spinal cord, but that ecotropic MuLV RNA is also found in smaller nonneuronal cells (Contag and Plagemann, 1988, 1989) (Fig. 5B). Severalfold more 3-kb message than full-length MuLV RNA has been generally found in the spinal cords of LDV-infected C58/M mice (Contag *et al.*, 1989; Contag and Plagemann, 1989) (Fig. 5A), as is typical for MuLV-infected cells (Varmus and Brown, 1989).

No clinical symptoms or overt pathological alteration in the spinal cord are associated with the expression of ecotropic MuLV RNA in spinal cord motor neurons (Contag *et al.*, 1989). Thus this process clearly differs from the motor neuron disease induced in newborn laboratory mice by infection with the ecotropic MuLV of wild mice (Cas-Br-E) (Gardner, 1989; Portis, 1990; Jolicoeur *et al.*, 1991), a temperature-sensitive variant of Moloney MuLV (Wong *et al.*, 1989; Wong, 1990), or a rat-passaged Friend MuLV (Kai and Furata, 1984).

Although the strong correlation between the expression of ecotropic MuLV RNA in spinal cord motor neurons and their susceptibility to cytocidal LDV infection suggests a causal relationship, the molecular mechanism involved has not been resolved. This relationship must be unique for motor neurons, because ecotropic MuLV RNA is found in cells other than motor neurons in these mice without rendering them

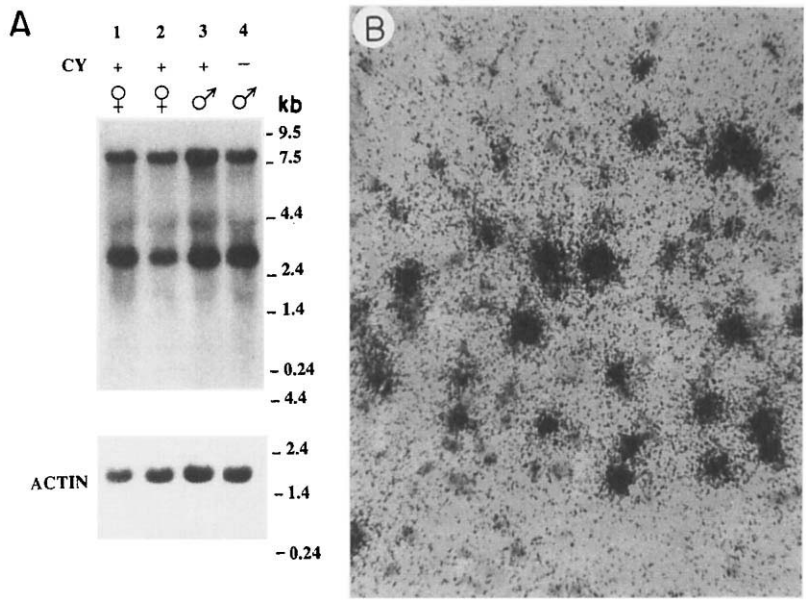

FIG. 5. Levels of ecotropic MuLV RNA in the spinal cords of 6-month-old C58/M mice (A) and cellular locations of the MuLV RNA in the spinal cord (B). Where indicated, individual mice were injected with cyclophosphamide (CY +; 200 mg/kg); 1 day later RNA was extracted from their spinal cords and analyzed by Northern blot hybridization using a ^{32}P-labeled, 27-mer oligonucleotide probe specific for the gp70 *env* region of the ecotropic MuLV genome and subsequently with an actin-specific oligonucleotide probe (A). A spinal cord section of a cyclophosphamide-treated 6-month-old C58/M mouse was hybridized with an ^{125}I-labeled, full-length proviral clone AKR-623 (B). [Results from studies by Contag and Plagemann (1989) and G. A. Palmer *et al.* (unpublished observations 1991).]

permissive to LDV infection. Outside the spinal cord, LDV replication in C58/M mice, as in other mouse strains, seems to be restricted to a subpopulation of macrophages. Thus it has been suggested that the susceptibility of the motor neurons to infection by LDV is mediated by a neuron-specific factor that is coincidentally expressed with MuLV RNA synthesis (Contag and Plagemann, 1989; Contag *et al.*, 1989). Perhaps a MuLV protein or a MuLV-induced neuronal surface protein acts as a surrogate receptor for LDV in neurons. Antibody protection studies (see later) suggest that such an LDV receptor would have to be different from that present on permissive macrophages (Contag *et al.*, 1989). Such a mechanism could explain a genetic linkage between ecotropic MuLV expression and susceptibility to ADPM.

However, some paradoxes need to be resolved that concern the mechanisms involved in the age-dependent expression of ecotropic MuLV RNA, specifically in spinal cord motor neurons, and in the genetic linkage between the Fv-$1^{n/n}$ genotype and ADPM susceptibility. The synthesis of ecotropic MuLV RNA in motor neurons could reflect proviral induction in these cells, perhaps as a result of age-related damage to motor neuron DNA, because DNA damage is known to activate endogenous provirus expression (Pincus, 1980; Varmus and Brown, 1989). Expression might be activated by provirus demethylation during DNA repair synthesis, because methylation is known to suppress MuLV provirus expression (Hoffman *et al.*, 1982; Coffin, 1985, 1990). However, such provirus induction should not be subject to regulation by the FV-$1^{n/n}$ locus, because Fv-1 restriction is due to an inhibition of an early step in the retroviral cycle. The Fv-1 product is postulated to inhibit the formation of appropriate forms of double-stranded DNA for integration by an interaction with the $p30^{gag}$ MuLV protein of an infecting virus (Yang *et al.*, 1980; Jolicoeur and Rassart, 1980; Des-Groseillers and Jolicoeur, 1983; Ou *et al.*, 1983; Coffin, 1990). Alternately, MuLV RNA synthesis in spinal cord motor neurons could reflect an age-dependent increase in infection of these cells by an N-tropic, ecotropic MuLV produced in other tissues or by nonneuronal cells in the spinal cord, perhaps a MuLV recombinant with enhanced capacity to infect motor neurons. The replication of the ecotropic MuLV in other tissues is clearly under the control of the *Fv-*$1^{n/n}$ locus and so would be the infection of motor neurons by exogenous MuLV. However, there is a question as to what extent retroviruses can infect motor neurons, because productive C-type retrovirus infection is thought to occur only in replicating cells (Varmus and Brown, 1989) and motor neurons are quiescent. The host cell factors or processes required for MuLV replication have not been identified, except for the requirement of host DNA repair enzymes for retrovirus integration (Coffin, 1990). Host DNA repair synthesis seems to be functional in motor neurons (Bradley and Krasin, 1982) and is perhaps sufficient for infection of motor neurons and other nonreplicating cells by MuLV. Indeed, certain strains of human immunodeficiency virus replicate well in nondividing human peripheral macrophages/monocytes (Melzer *et al.*, 1990). On the other hand, in the case of Cas-Br-E-infected mice, viral RNA has been detected by *in situ* hybridization only in nonneuronal cells, especially astrocytes, in the spinal cord, and the degeneration of motor neurons has been postulated to be caused indirectly by the infection of the nonneuronal cells (Jolicoeur *et al.*, 1991). Aberrant retrovirus-like particles have been detected in motor neurons of Cas-Br-E-infected mice, but the origin of these particles is uncertain (Portis, 1990).

Preliminary experiments have shown that the envelope MuLV glycoprotein 70 (gp70) is expressed in motor neurons of 6- to 7-month-old C58/M mice synthesizing MuLV RNA (Contag and Plagemann, 1989), but attempts to isolate infectious MuLV from the spinal cords of these mice have failed, even though infectious MuLV could be readily isolated from the spleens of these animals as well as from the spinal cords of 21-day-old Swiss mice that were infected with Cas-Br-E MuLV at 1 day of age (P. G. W. Plagemann *et al.*, unpublished observations). MuLV particles were also not detectable by electron microscopy in spinal cord sections of 6- to 7-month-old C58 mice (Brinton *et al.*, 1986b; G. A. Palmer and P. G. W. Plagemann, unpublished observations 1991). The results may suggest that the synthesis of ecotropic MuLV RNA and protein in the motor neurons is not associated with the formation of infectious virus particles.

In initial studies (Contag and Plagemann, 1989), cyclophosphamide treatment was found to increase transiently the expression of ecotropic MuLV RNA in spinal cord neurons of 6- to 7-month-old C58/M mice and it was suggested that this effect plays a role in increasing the susceptibility of these mice to ADPM (Table II). However, in subsequent experiments, cyclophosphamide treatment had no effect on the level of ecotropic MuLV RNA in spinal cords of 6- to 7-month-old C58/M mice (G. A. Palmer *et al.*, unpublished observations 1991) (Fig. 5A). Instead it was apparent that cyclophosphamide treatment generally increases ADPM susceptibility of mice already expressing significant levels of MuLV RNA by suppressing a neuron-protective anti-LDV immune response (see below).

4. Specific Protection of Motor Neurons against LDV Infection by Anti-LDV Immune responses

a. Protection by Endogenously Generated Immune Response. C58/M mice generally do not develop paralytic disease after infection with a neurovirulent strain of LDV until they reach 10–12 months of age, and even in these old mice the frequency of ADPM is variable and relatively low (Murphy *et al.*, 1983; (Table II). However, Murphy *et al.* (1983) showed that C58/M mice 7 months of age and older become highly sensitive to ADPM when X-irradiated (550–600 rads) 1 day before LDV infection, but that up to 4 months of age the mice are highly resistant, even when X-irradiated. Similar observations have been made with C58/M mice that received a single injection of cyclophosphamide (200 mg/kg) 1 day before infection with neurovirulent LDV (Table II).

It has become apparent, however, that the relative resistance of young cyclophosphamide-treated C58/M mice to ADPM is not absolute

and partly reflects their ability to recover from the immunosuppressive treatment and develop an anti-LDV response before paralytic disease develops. It was observed that the cyclophosphamide treatment 1 day before LDV infection suppresses an anti-LDV immune response in most C58/M mice only for 12–20 days (Cafruny *et al.*, 1986c; P. G. W. Plagemann *et al.*, unpublished observations). The same is probably the case for sublethal X-irradiation (Onyekaba *et al.*, 1989a). When examined 20–40 days postinfection, most cyclophosphamide-treated C58/M mice not developing paralysis, regardless of age, have been found to possess plasma LDV antibody levels that approached those of companion mice that had not been treated with cyclophosphamide. The same was true for about 30–50% of paralyzed 6- to 7-month-old mice at the time of paralysis, whereas no anti-LDV antibodies were detected in the other paralyzed mice (Cafruny *et al.*, 1986c; P. G. W. Plagemann *et al.*, unpublished observations).

The results indicate that some C58/M mice recover from a single administration of cyclophosphamide 1 day before LDV infection and mount an anti-LDV immune response soon enough to block the progressive spread of the LDV infection between motor neurons before a sufficient number of them are destroyed to cause frank paralysis. This hypothesis is in agreement with the detection of a limited number of LDV-infected motor neurons in the spinal cords of cyclophosphamide-treated 2- to 3-month-old C58/M mice 8–10 days postinfection (Brinton *et al.*, 1986b). Furthermore, it is supported by the finding that 1- to 5-month-old C58/M mice, though highly resistant to ADPM after a single cyclophosphamide treatment, become sensitive when cyclophosphamide is administered repeatedly, once prior to LDV infection and thereafter in 7- or 10-day intervals (Table II). None of the mice receiving multiple injections of cyclophosphamide developed anti-LDV antibodies (P. G. W. Plagemann *et al.*, unpublished observations), just as is the case in Swiss or BALB/c mice (Onyekaba *et al.*, 1989a). The latent period in these young mice, however, was relatively long (Table II), perhaps because the low level of expression of ecotropic MuLV in the motor neurons allows only a slow spread of the LDV infection between motor neurons and thus delays their destruction.

Differences in the efficiency of mounting an anti-LDV immune response after a single administration of cyclophosphamide might also account for apparent differences in susceptibility of mice from different colonies of C58 or AKR mice (substrains). For example, though 6- to 8-month-old C58/M mice became highly susceptible to ADPM after a single injection of cyclophosphamide before LDV infection, only relatively few 6- to 8-month-old C58/J mice developed paralytic disease

under identical conditions (Table II). Similar differences were observed for AKR/Boy mice on the one hand and AKR/NIH and AKR/J mice on the other (Table II). However, 6- to 8-month-old AKR/J mice became susceptible after multiple injections of cyclophosphamide (Table II), which completely blocked the development of an anti-LDV immune response. AKR/J mice at 6 months of age also required higher doses of X-radiation to render them susceptible to ADPM than did C58/M mice (Pease *et al.*, 1982), and the relative resistance of immunosuppressed 6-month-old C58/J and AKR/J mice to ADPM was lost in older mice (Pease and Murphy, 1980). A difference in the immune response between individual substrains of AKR or C58 seems surprising because these substrains are genetically identical, except for slight differences in proviral content (see later). Alternately, therefore, it is possible that the differences in relative sensitivity of the substrains to ADPM is, at least partly, related to the expression of specific ecotropic proviruses in these substrains or that their expression affects the immune response of the mice.

The importance of the immune response in protection against ADPM is also indicated by the finding that 2-month-old nude AKR/J mice, which failed to produce anti-LDV antibodies, developed ADPM whether or not they were treated with cyclophosphamide 1 day before LDV infection, whereas their heterozygous and wild-type littermates were resistant under these conditions (Table II). The latter, however, became paralyzed when receiving multiple injections of cyclophosphamide, just as did 2-month-old C58/M mice (Table II). Thymectomy at birth of C58 mice also enhanced their susceptibility to ADPM (Duffey *et al.*, 1976).

It has been suggested that the susceptibility to ADPM may be controlled by a gene linked to the H-2 complex because AKR H-2^b/Boy (H-2^b from C57BL/6Boy) and AKR/M (*H-2*^{m}) mice at 6 months of age were resistant to ADPM after a single cyclophosphamide treatment, whereas *H-2*^{k} AKR mice from various colonies were sensitive (Martinez, 1979). Although subsequent genetic studies did not support this view (Pease *et al.*, 1982), an unexplained complex pattern of susceptibility to ADPM emerged among various F_1 hybrid and backcross populations. It seems likely that these differences were also related to differences in the efficiency with which various strains and their hybrids overcame the suppression of the anti-LDV immune response by a single immunosuppressive treatment before LDV infection. This view is supported by the finding that, although none of the immunosuppressed 6-month-old AKR *H-2*^{b}/Boy mice developed paralytic disease after LDV infection, 5/6 12-month-old mice did (Murphy *et al.*, 1987). Simi-

larly, the maternal effect on ADPM susceptibility mediated by the $H\text{-}2^b$ haplotype (Pease *et al.*, 1982) could be linked to differences in the efficiency of the mice to mount a protective anti-LDV immune response.

Overall, the results point to the importance of an anti-LDV immune response in protecting C58 and AKR mice from the development of paralytic disease after LDV infection (see Fig. 3). However, it is unclear at present to what extent the motor neuron protection is mediated by anti-LDV antibodies or CTLs, because on passive transfer both are protective (see Sections II, B, 4, b and II,, B, 4, c). Regardless, it should be emphasized that motor neurons are protected by the anti-LDV immune response without affecting LDV replication in permissive macrophages (Harty *et al.*, 1987b; Harty and Plagemann, 1990). The efficiency of mounting a motor neuron-protective anti-LDV immune response seems to decrease with increase in age of the mice and so does the efficiency with which mice recover from a single immunosuppressive treatment (X-irradiation or cyclophosphamide). The efficiency of recovery also varies between individual mice and between different strains of AKR and C58 mice, and may perhaps be partly influenced by the *H-2* haplotype of these mouse strains. One conclusion arises, namely, that the apparent susceptibility of mice to ADPM is a function of at least two factors: the presence and expression of endogenous ectropic MuLV in motor neurons and the efficiency of the anti-LDV immune response that specifically protects these motor neurons from LDV infection. Under the conditions of most earlier experiments the contributions of the two factors could not be adequately distinguished. To assess the contribution of each, it seems advisable to compare the incidence of paralytic disease induced by LDV infection in nonimmunosuppressed mice and in companion mice in which the anti-LDV response is continuously blocked by repeated administration of X-irradiation or cyclophosphamide in 10-day intervals. Another approach would be to use nude AKR mice.

Another potential complicating factor is that the incidence of ADPM in C58/M mice has been found to be also enhanced by increasing the size of the initial inoculum of neurovirulent LDV (Murphy *et al.*, 1983). For example, X-irradiated, 4-month-old C58/M mice were resistant to ADPM when injected with 10^2 ID_{50} of LDV/mouse, whereas over 90% of the mice developed paralysis when injected with 10^6–10^8 ID_{50} of LDV/mouse. A similar LDV dose effect has been observed by Harty *et al.* (1987b), but only below 10^4 ID_{50}/mouse. The mechanism of this LDV dose effect is not understood. Because plasma LDV titers reach similar levels in mice at 1 day postinjection (10^9–10^{10} ID_{50}/ml), whether the

mice are inoculated with 10^2 or 10^6 ID_{50}/mouse, the results suggest that LDV enters the spinal cord only very early after peripheral injection because at later times entry becomes blocked, perhaps by interferon that is rapidly induced by LDV infection. It also suggests that the efficiency of LDV entry into the spinal cord and initial infection of spinal cord motor neurons is very low and a function of the LDV concentration in the periphery. One needs to recall that an efficient infection of cultured peritoneal macrophages *in vitro* requires a m.o.i. of at least 100 ID_{50}/cell. Though the initial infection of spinal cord motor neurons can be blocked by anti-LDV antibodies (see below), their formation in LDV-infected mice is not rapid enough to account for the LDV dose effect discussed above.

The resolution of these questions requires a better understanding of how LDV gains entry into the CNS. Results from preliminary experiments indicated that injection of susceptible C58/M mice with LDV in the footpads of front or hind limbs resulted in the preferential development of paralysis in the injected limbs (Contag and Plagemann, 1989). These results were consistent with invasion of the CNS via retrograde axonal transport, as observed for other neurotropic viruses (Johnson, 1982; Tyler *et al.*, 1986). However, in further experiments no correlation was observed between the individual limbs of C58/M mice that were inoculated with LDV and the limbs that showed the first signs of paralysis (G. W. Anderson and P. G. W. Plagemann, unpublished observations).

b. Protection by Passive Immunization with Polyclonal and Monoclonal Anti-LDV Antibodies. The protective effect of anti-LDV antibodies against ADPM was first indicated by the finding that passive immunization of 6- to 8-month-old C58/M mice, which had been rendered susceptible to ADPM by an immunosuppressive treatment 1 day before LDV infection, with serum from LDV-infected mice (IMP) prevented the development of paralytic disease in these mice (Murphy *et al.*, 1983). This finding has been confirmed (Table III) and the protection has been shown to be mediated by nonneutralizing and probably neutralizing anti-LDV antibodies formed in mice during a natural infection (Harty *et al.*, 1987a, b; Harty and Plagemann, 1990). The protective anti-LDV antibodies are directed toward the envelope glycoprotein of LDV, VP-3 (Harty *et al.*, 1987b). In an extension of these studies it was shown that nonneutralizing MAbs raised to glutaraldehydyde-inactivated LDV, though also directed to LDV VP-3, failed to protect C58/M mice under similar conditions (Harty *et al.*, 1987a). Nonneutralizing, VP-3-specific anti-LDV MAbs raised to formalin-inactivated

TABLE III

PROTECTION OF C58/M MICE FROM ADPM BY POLYCLONAL AND MONOCLONAL ANTI-LDV ANTIBODIES[a]

Anti-LDV antibody	Time of administration of anti-LDV (days postinfection)	Paralyzed/ total mice	Paralyzed mice (%)	Mean onset of paralysis ± SEM (days postinfection)
None		15/15	100	16 ± 0.9
IMP (polyclonal)	−1	3/21	14	24 ± 1.2
Neutralizing MAb	−1	5/43	12	26 ± 1.5
Neutralizing MAb	+0.5, +2, or +4	22/29	76	24 ± 1.2
Neutralizing MAb	+2, +4, and +7	2/18	11	25 ± 2.5
Nonneutralizing MAb	−1	18/20	90	15 ± 1.0

[a] The results are compiled from Harty and Plagemann (1990). Groups of 6- to 7-month-old C58/M mice were treated with 200 mg cyclophosphamide/kg, to render them susceptible to ADPM, 2 days before i.p. injection of 10^6 ID_{50} of a neurovirulent strain of LDV (LDV-v). As indicated, the mice were also injected intravenously with 0.5 ml of 1 : 2 or 1 : 3 dilutions of plasma from LDV-infected mice (IMP) or of ascites fluid containing anti-LDV neutralizing MAb (159-7, −12, −16, −18, or −19) or nonneutralizing MAb (159-3, −4, −5, −9, or −14) generated to formalin-inactivated LDV. The mice received *single* injections of antibodies 1 day before infection (−1 day postinfection) or 0.5, 2, or 4 days postinfection, or they received *multiple* injections at 2, 4, and 7 days postinfection. The mice were observed for paralysis at least until 45 days postinfection.

LDV that recognize at least three different epitopes were also nonprotective (Harty and Plagemann, 1990) (Table III). In contrast, passive immunization with five neutralizing MAbs to formalin-inactivated LDV 1 day before LDV infection afforded efficient protection (Harty and Plagemann, 1990) (Table III). These neutralizing MAbs recognize a single epitope on VP-3 to which no antibodies are generated during a natural infection (see Section II, A, 2b). Thus, antibodies to at least two epitopes of LDV VP-3 afford neuron protection, one recognized by the neutralizing MAbs and one or more recognized by polyclonal anti-LDV formed during a natural infection of mice.

In situ hybridization analyses have shown that the polyclonal and monoclonal protective antibodies specifically protect motor neurons from LDV infection; no LDV RNA containing motor neurons were detectable in spinal cord sections of antibody-protected mice (Harty *et al.*, 1987b; Harty and Plagemann, 1990). On the other hand, LDV RNA was detected in an apparently undiminished number of smaller nonneuronal cells in these spinal cord sections. These cells might be microglial cells or macrophages that became infected via the hematogenous

route or entered the CNS through the blood–brain barrier, respectively (Fig. 3). In this context, it should be recalled that the neuron-protective anti-LDV antibodies do not protect permissive macrophages from infection by LDV.

Interestingly, passively transferred neutralizing anti-LDV MAbs can protect motor neurons in susceptible C58/M mice at two temporally distinct stages in the development of ADPM (Harty and Plagemann, 1990) (Table III and Fig. 3). When administered 1 day before or at the time of LDV infection of susceptible C58/M mice, they seem to prevent the initial infection of motor neurons in the spinal cord. When administered in a single equivalent dose 0.5–4 days after LDV infection, on the other hand, they afford little protection, though the immunization seems to delay the onset of paralytic disease (Table III). Protection, however, can be achieved after infection by repeated injections of the protective antibodies (Table III). The results indicate that protection by antibodies administered postinfection is mediated by transiently blocking the spread of the cytocidal LDV infection of motor neurons in a fashion similar to the protective effect of the anti-LDV immune response generated naturally in nonimmunosuppressed mice. In immunosuppressed susceptible mice that normally develop paralytic disease, repeated injections of protective antibodies are required until the mice develop their own anti-LDV immune response on recovery from the single immunosuppressive treatment (Fig. 3). In agreement with this hypothesis, a second administration of cylophosphamide at 7 days postinfection abolished the protection achieved by giving protective antibodies at 2, 4, and 7 days postinfection (Harty and Plagemann, 1990).

The mechanism by which anti-LDV antibodies that recognize specific epitopes on LDV VP-3 protect motor neurons from LDV infection has not been resolved. However, as discussed already, the finding that the antibodies protect neurons without interferring with the infection of permissive macrophages suggests that they function by a cell type-specific (neuron-specific) neutralization of LDV infectivity, perhaps at the level of a surrogate LDV receptor on motor neurons induced by MuLV expression.

c. Protective T Lymphocytes. An increase in expression of ecotropic MuLV in motor neurons seems to be one factor that increases the susceptibility of C58 and AKR mice to ADPM with increase in age. A second factor is a decline in the functioning of the host immune system, which is indicated by two lines of evidence. First, the requirement of immunosuppressive treatments to render C58 mice susceptible to ADPM decreases with age (Table II). Multiple cyclophoshamide treatments are required to render young C58 mice susceptible, whereas 14-

to 20-month-old C58 mice, like nude AKR mice, are susceptible without immunosuppressive treatment. Second, it has been shown that passive transfer of spleen cells from uninfected young, but not old, C58 mice to old immunosuppressed C58 mice protects the latter from ADPM (Murphy *et al.*, 1980; Duffey *et al.*, 1976; Bentley and Morris, 1982). On the other hand, injection of young C58 mice with spleen cells from old C58 mice does not render the former susceptible to ADPM. In addition, some C58 mice, when infected with LDV at 1 month of age, develop ADPM when they are 12–15 months of age, if they have not earlier succumbed to leukemia (Murphy *et al.*, 1983).

The function of the T cell population that is lost with age in C58 mice in protection from ADPM, has not been elucidated. Lawton and Murphy (1973) reported that the ability of spleen cells of C58 mice to be activated by phytohemagglutinin decreases about 50% between 4 and 16 months of age, but the reasons for this change were not explored. It is probably not a Th cell population (required for the production of protective anti-LDV antibodies) that is lost, because (1) the ability of C58 mice to produce antibodies to sheep red cells does not become impaired with age (Murphy *et al.*, 1987; Cafruny *et al.*, 1986c) and (2) the plasma anti-LDV antibody titers of nonimmunosuppressed 17- to 20-month-old C58 mice at the time of paralysis (Table II) were as high as those developed by young nonimmunosuppressed C58 mice that are resistant to ADPM (P. G. W. Plagemann *et al.*, unpublished observations). Although these findings indicate that the ability of C58 mice to mount a humoral antibody response does not become quantitatively impaired with age, in neither case cited above has it been demonstrated that the antibodies formed by old C58 mice are qualitatively identical to those formed by young C58 mice.

Nevertheless, it seems more likely that the protective T cells that are lost with age have some other as-yet unidentified function. Bentley and Morris (1982) reported that the protection of immunosuppressed 5- to 6-month-old C58 mice by spleen cells from uninfected 4- to 6-week-old C58 mice required all three distinct types of T cells, Lyt-1, Lyt-2 ($CD8^+$), and Lyt-1,2 T cells. In another study, Bentley *et al.*, (1983) reported that Lyt-1,2 T cells alone were sufficient to protect 11-month-old C58 mice that were naturally susceptible to ADPM. It was suggested that Lyt-1,2 cells represent undifferentiated precursor cells, perhaps suppressor T (Ts) cells and that it is this population that becomes lost with age in C58 mice. On the other hand, Lyt-2 cells, but not Lyt-1 cells, from 3-day LDV-infected young C58 mice ("sensitized T cells") alone were able to protect immunosuppressed old C58 mice from ADPM. The results suggest that protection is mediated by CTLs or Ts

cells, and that $CD8^+$T lymphocytes represent the population of spleen cells that is lost with age in C58 mice. The protective lymphocytes could be LDV-specific CTLs that specifically lyse LDV-infected motor neurons and thereby arrest the spread of the LDV infection between motor neurons. However, CTLs lyse target cells only in the context of the MHC class I surface antigen (Whitton and Oldstone, 1990), and the level of expression of class I or class II antigens on neurons is known to be very low, which makes them relatively resistant to CTL attack (Oldstone *et al.*, 1986; Hickly and Kimura, 1988; Joly *et al.*, 1991). Perhaps, therefore, LDV replication in motor neurons is suppressed by CTLs indirectly by a noncytopathic effect mediated by lymphokines, as has been postulated for the clearance of lymphocytic choriomeningitis virus (LCMV) from the central nervous system (Oldstone *et al.*, 1986; Klavinski *et al.*, 1989; Whitton and Oldstone, 1990). IFN-γ known to be produced by CTLs (Mossmann and Coffman, 1989), has been implicated in the clearance of LCMV from tissues by LCMV-specific CTLs in mice (Klavinskis *et al.*, 1989; Leist *et al.*, 1989). Clearly further work is required to define the nature of the motor neuron-protective T cell population of C58 mice, its function in ADPM resistance, and why it becomes lost in these mice with age.

5. *Relationship between Ecotropic Proviral Content and ADPM Susceptibility*

Initial experiments suggested that the susceptibility of mouse strains to ADPM is dependent on the presence of multiple endogenous ecotropic proviruses (Pease and Murphy, 1980; Murphy *et al.*, 1983). For example, at least three ecotropic proviruses are found in AKR and PL/J mice and six are found in C58 mice (Fig. 6), which are all susceptible (Coffin, 1984, 1985; Kozak, 1985). On the other hand, C3H/Hej, DBA/2J, and C57BL/6*Fv-1*^{n}/Boy mice have been reported to be resistant to ADPM at 12 months of age, even though each possesses one ecotropic provirus, emv-1, emv-3, or emv-2, respectively (Jenkins *et al.*, 1982; Coffin, 1984, 1985), and the *Fv-1*$^{n/n}$ haplotype, and the mice were X-irradiated before LDV infection (Pease and Murphy, 1980). The most likely explanation for the resistance of these mouse strains to ADPM is that their proviruses carry minor defects and are therefore not expressed (Coffin, 1985). For example, little MuLV is produced in *Fv-1*$^{n/n}$ mouse strains that carry either the emv-1 or emv-2 provirus, but MuLV production is greatly enhanced in F_1 hybrids of these strains, probably as a result of the generation of infectious recombinants between the two proviruses (Coffin, 1985). The defect in emv-2 has been shown to be caused by a single base substitution in codon 3 of the gene sequence encoding pis^{gag}

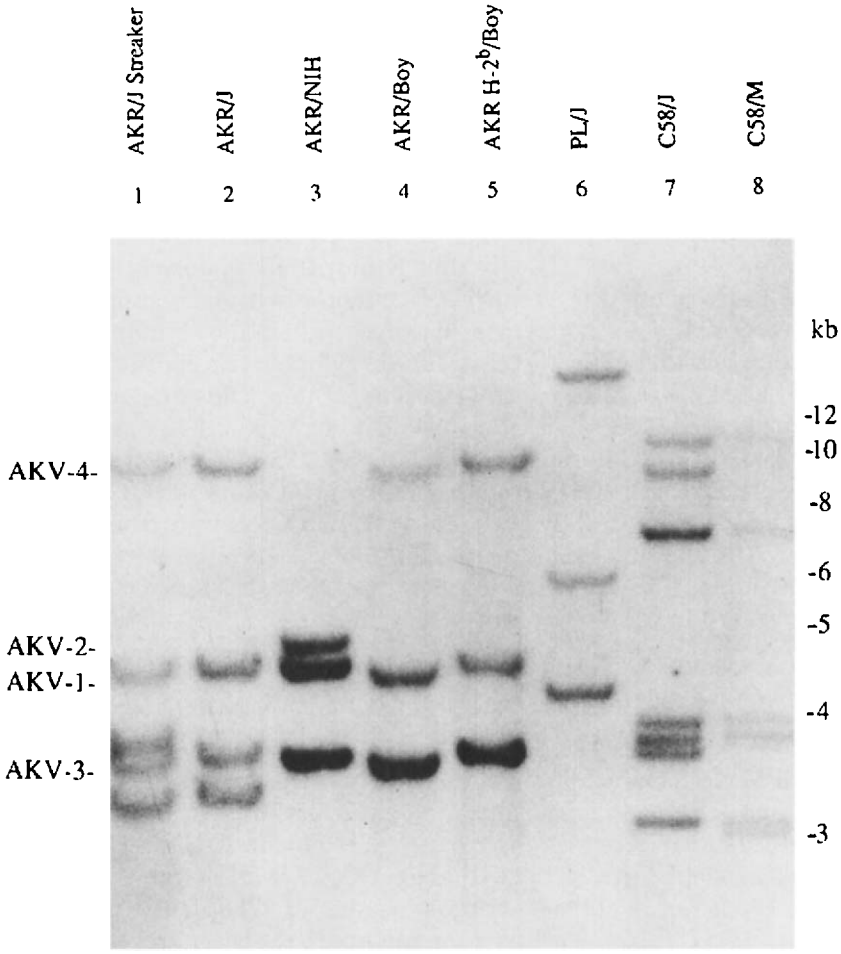

FIG. 6. Content of ecotropic proviruses of chromosomal DNA isolated from different ADPM-susceptible substrains of mice. The DNA of the indicated mice was digested with *Pvu*II and was analyzed by Southern hybridization using a ^{32}P-labeled, 180-bp, *Sma*I–*Sma*I fragment of the *env* gene of AKR-623 as probe (G. W. Anderson and P. G. W Plagemann, unpublished observations 1991). AKV-1 to AKV-4 have been described previously (emv-11 to emv-14, respectively) (Coffin, 1984; Jenkins *et al.*, 1982).

(Copeland *et al.*, 1988; Mercer *et al.*, 1990). The resulting amino acid substitution inhibits myristylation of the gag precursor and thus virus assembly. There is also evidence that the various proviruses of AKR mice are not identical and that the emv-13 (AKV-3) provirus (Fig. 6) is defective (Coffin, 1985). Recent studies of inbred recombinant strains of

AKR/J and DBA/2J mice (Ak×D) that carry only single ecotropic proviruses of the AKR mice (Jenkins *et al.*, 1981) support the conclusion that ADPM susceptibility requires only the presence of a single nondefective ecotropic provirus that is expressed (G. W. Anderson and P. G. W. Plagemann, unpublished observations 1991). None of thee 7-month-old AK×D mice carrying only the defective emv-13 provirus (Ak×D-28) developed paralytic disease after LDV infection, even though the mice were continuously immunosuppressed by repeated injections of cyclophosphamide. In contrast, Ak×D mice carrying only the nondefective prototype emv-11 provirus of AKR mice (Ak×D-16) developed paralytic disease under the same conditions.

Superimposed on the effect of defectiveness on ecotropic provirus expression and ADPM susceptibility is the possibility that only certain ecotropic proviruses become expressed in motor neurons or that only certain ecotropic proviruses can generate recombinants that can infect motor neurons. These possibilities can be experimentally examined. The number of ecotropic proviruses in 17 substrains of AKR mice that have been analyzed varied between two and six, and only one ecotropic provirus, that present in the original strain of AKR mice (emv-11, AKV-1), was shared by all the substrains (Coffin, 1985) (Fig. 6). Other ecotropic proviruses are shared by certain AKR substrains, such as the defective emv-13/AKV-3 by AKR/J, AKR/Boy, and AKR/NIH mice, and emv-14/AKV-4 by AKR/J and AKR/Boy mice (Fig. 6). The AKR ecotropic proviruses also differ from those found in the susceptible C58 and PL/J mice. C58/M and C58/J mice also share five, but not all, ecotropic proviruses (Fig. 6). Thus more than one nondefective ecotropic provirus may function in rendering mice susceptible to ADPM in context with the *Fv-1$^{n/n}$ haplotype*.

6. Synopsis

The paralytic motor neuron disease induced by LDV infection in certain mouse strains exhibits a multifactorial etiology. It is dependent on three factors (Fig. 3): (1) A genetic predisposition that is linked to the presence of at least one nondefective ecotropic, N-tropic MuLV provirus, and the Fv $1^{n/n}$ genotype, which permits expression of the provirus. Expression of the ecotropic provirus or a recombinant arising from this provirus in anterior horn motor neurons renders these cells susceptible to cytocidal infection by LDV. (2) An acute infection with a neurovirulent strain of LDV. Upon infection of a mouse, LDV will infect a permissive subpopulation of macrophages but will also rapidly invade the CNS and infect a limited number of susceptible motor neurons. Infection of these motor neurons must occur early after the primary infection of the mouse because it seems to be blocked within the first

day postinfection by an as-yet undefined mechanism. From initial foci of infection in the spinal cord, the cytocidal LDV infection spreads progressively to other neurons in the infected area until the number of cells destroyed is sufficient to cause frank paralysis (generally 12–30 days postinfection). (3) Suppression of the host anti-LDV immune response to LDV infection, because the latter specifically blocks the cytocidal infection of motor neurons by LDV without affecting LDV replication in macrophages. Motor neuron protection is mediated both by anti-LDV antibodies to at least two epitopes of the envelope glycoprotein of LDV and a population of protective T lymphocytes, most likely $CD8^+$ cells.

The susceptibility of genetically predisposed mice increases progressively with increase in age due to an increased expression of ecotropic MuLV in the motor neurons, which renders them susceptible to LDV infection, in combination with an age-dependent loss of the T cell population that protects the motor neurons from LDV infection.

It seems possible that a similar complex interaction between endogenous retroviruses, a second cytocidal virus, and dysfunction of the host immune system plays a role in the etiology of human age-related central nervous system diseases. The human DNA genome carries a multitude of human endogenous retroviruses (HERVs) (Larsson *et al.*, 1989). One family of HERVs is related to mammalian C-type viruses (Larsson *et al.*, 1989) Three single-copy proviruses have been isolated as well as some proviruses that are present in multiple copies, up to 1000/haplotype (Larsson *et al.*, 1989; Mariani-Constantini *et al.*, 1989). Though all the proviruses that have been sequenced are defective, some carry nondefective retrovirus genes that can be expressed (Larsson *et al.*, 1989; Wilkinson *et al.*, 1990). Furthermore, immunocompetence in the elderly generally becomes impaired to varying degrees (Chopra, 1990; Ennis, 1990).

C. Molecular Properties

1. Stucture, Composition, and Stability of Virion

LDV is a spherical, enveloped virus with a diameter of 50–55 nm and a nucleocapsid core of 30–35 nm (Brinton-Darnell and Plagemann, 1975; Horzinek *et al.*, 1975) (Table I). Virions exhibit a relatively low buoyant density in both sucrose and glycerol density gradients (1.13–1.14 g/cm^3) (Darnell and Plagemann, 1972; Brinton-Darnell and Plagemann, 1975; Michealides and Schlesinger, 1973; Horzinek *et al.*, 1975) and a sedimentation coefficient of 204 S (Horzinek *et al.*, 1975) or 230 S (Plagemann *et al.*, 1991) as estimated by comparison to that of Semliki

Forest virus (SFV; 264 S) (Horzinek *et al.*, 1975). However, the S value for LDV must be considered a rough estimate because of uncertainties as to the accuracy of the S value for SFV (Horzinek, 1973; Westaway *et al.*, 1985a). Infectious LDV is relatively unstable. Infectivity in plasma is stable at −70°C, but slowly lost at −20°C (tl/2 = about 4 weeks) (P. G. W. Plagemann, unpublished observations) and more rapidly at temperatures above 0°C (Rowson and Mahy, 1985). Treatment of virions with nonionic detergents, such as Nonidet P-40 (NP-40) or Triton X-100, even at concentrations as low as 0.01%, release free nucleocapsids (Michaelides and Schlesinger, 1973; Brinton-Darnell and Plagemann, 1975). The nucleocapsids have a buoyant density of about 1.17 g/cm^3 (Brinton-Darnell and Plagemann, 1975) and a sedimentation coefficient of about 176 S (Horzinek *et al.*, 1975). Drying and/or changes in salt concentration used in the preparation of purified virus for electron microscopy generally cause disintegration of the virions, with sloughing off of the viral envelope and the occasional formation of 8- to 14-nm-diameter hollow ringlike particles (Brinton-Darnell and Plagemann, 1975). The latter particles probably represent envelope subunits because similar structures can be distinguished on the surface of intact LDV particles (Brinton-Darnell and Plagemann, 1975) and on the surface of EAV (Horzinek, 1981). The envelope of flaviviruses is also composed of ringlike structures, but these are somewhat smaller (about 7nm) (Murphy, 1980).

The LDV nucleocapsid is composed of the nucleocapsid protein, VP-1 (13–15 kDa) (Michaelides and Schlesinger, 1973; Brinton-Darnell and Plagemann, 1975; Coutelier et al., 1986), and the 48 S single-stranded RNA genome (12–13 kb) with positive polarity (Darnell and Plagemann, 1972; Brinton-Darnell and Plagemann, 1975) and a 3'-terminal poly (A) sequence of varying length (Contag *et al.*, 1986b; Brinton *et al.*, 1986a). The envelope contains two LDV proteins, a nonglycosylated protein, VP-2 (17–19 kDa), and a glycosylated protein, VP3, which varies in size from 24 to 44 kDa (Michaelides and Schlesinger, 1973; Brinton-Darnell and Plagemann, 1975; Coutelier *et al.*, 1986). Up to 10 distinct molecules have been identified in the VP-3 molecular range (Cafruny *et al.*, 1986a). These probably represent a single protein that is glycosylated to different extents because various anti-LDV antibodies that recognize at least three different epitopes each seem to interact with all molecular species of VP-3 (Harty and Plagemann, 1988). The relative smallness of the envelope glycoprotein(s) of LDV probably explains the smoothness of the surface of the virions (Brinton-Darnell and Plagemann, 1975; Stueckemann *et al.*, 1982a; Horzinek *et al.*, 1975) and perhaps the lack of hemagglutination activity of LDV (Rowson and Mahy, 1985).

2. Virion RNA and Genomic Organization

cDNAs have been generated from the RNA of two different LDV isolates (LDV-C and LDV-P; see Section II,C,3) by reverse transcription using oligo(dT) and calf thymus DNA-derived random primers (Contag *et al.*, 1986a; Godeny *et al.*, 1989, 1990; Kuo *et al.*, 1991). Specificity of the cDNAs has been ascertained by Northern blot hybridization of RNA extracted from purified virus, LDV-infected mouse macrophage cultures, or tissues from LDV-infected mice (Fig. 4). A partial sequence consisting of the 1064 3'-terminal nucleotides of LDV-C has been published (Godeny *et al.*, 1989, 1990). A 3'-terminal noncoding sequence of 80 nucleotides is preceded by two complete open reading frames (ORFs), which are in different reading frames and overlap by 11 nucleotides. The most 3' ORF encodes a 115-amino acid protein (12.2 kDa) that has been identified as VP-1 by sequencing the N-terminal 33 amino acids of purified VP-1 (Godeny *et al.*, 1990). The second ORF encodes a 171-amino acid protein that lacks glycosylation sites but possesses three potential membrane-spanning segments (L. Kuo *et al.*, unpublished observations 1991). Combined, these properties suggest that it is the nonglycosylated envelope protein of LDV, VP-2. Its ORF overlaps with a third incomplete adjacent ORF by 10 nucleotides. Sequence analysis of the 3'-terminal 2030 nucleotides of LDV-P (based on the cDNAs shown in Fig. 7B) indicates that the third ORF encodes a 265-amino acid protein (Kuo *et al.*, 1991 unpublished observations). Its function has not been identified, but on the basis of its size and the presence of four potential N-glycosylation sites, it could be the virion envelope glycoprotein, VP-3.

The location of the genes for structural viral proteins at the 3' end of the LDV genome is typical for alpha and rubi togaviruses, but the apparent overlap of the ORFs is not, and no significant sequence similarity between the 3' ends of LDV RNA and the RNAs of alpha and rubi togaviruses has been detected. Instead, it has been suggested (Godeny *et al.*, 1990), that the genome organization of LDV resembles that of EAV and thus those of coronaviruses and toroviruses (Spaan *et al.*, 1990). This suggestion has recently been supported by the identification of a 3'-coterminal nested set of seven subgenomic RNAs in LDV-infected macrophage cultures (Kuo *et al.*, 1991). Replication via 3'-coterminal nested sets of subgenomic mRNAs is one common characteristic of arteri-, toro-, and coronaviruses (Spaan *et al.*, 1990; den Boon *et al.*, 1991). The nested set of six subgenomic mRNAs of EAV ranges in size from about 0.8 to 3.2 kb (see later, Fig. 11). The 3'-coterminal nested set of seven RNAs of LDV ranges in size from about 1.0 to 3.6 kb (designated RNAs 2–8; Fig. 7A). The RNAs were detected by Northern

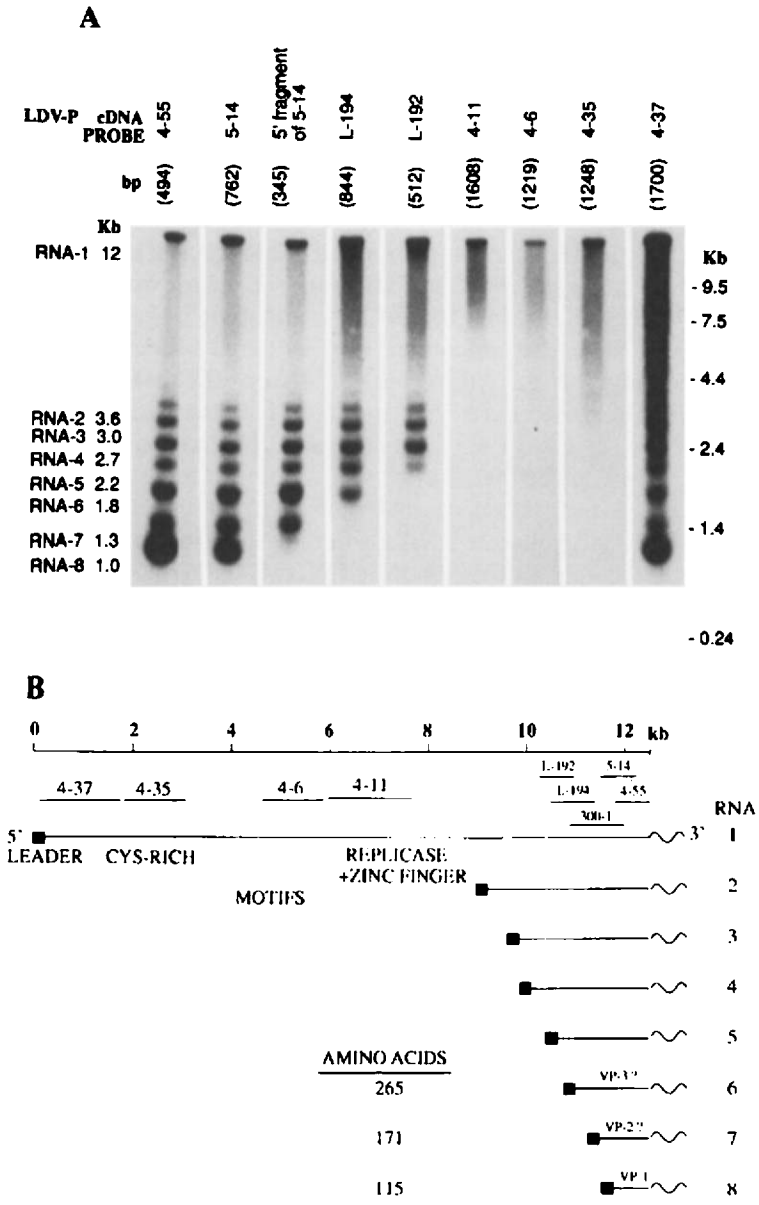

FIG. 7. Northern blot hybridization analyses of RNA isolated from 6-hr LDV-infected macrophage cultures using different LDV-P-specific cDNA probes (A) and tentative genome organization of LDV-P (B). A single blot of total cellular RNA was sequentially hybridized with the different cDNA probes (4-55, 5-14, L-192, L-194, 4-11, 4-6, 4-35, 4-37, and a 5'-*Hae*III restriction fragment of cDNA 5-14. The length of each cDNA is indicated in parentheses. (Data from Kuo *et al.*, 1991, and unpublished observations.) (B) The wavy lines indicate the 3'-poly (a) tails of the RNAs and the cross-hatched bars indicate segments of homology with EAV.

blot hybridization analyses of total RNA extracted from 6-hr LDV-infected macrophage cultures using a cDNA probe (4-55, 494 nucleotides) to the 3' end of the genome of LDV-P, which encompasses the entire VP-1 gene (Kuo *et al.*, 1991) (Fig. 7B). Another cDNA (5-14, 762 nucleotides), which overlaps cDNA 4-55 and encompasses the entire VP-2 ORF, also hybridizes to all eight subgenomic RNAs, whereas a 5' *Hae*III restriction fragment of cDNA 5-14 (345 nucleotides) hybridizes only to the six largest subgenomic RNAs (Fig. 7A). Two other cDNAs, L-192 and L-194, hybridize to the four and five largest subgenomic RNAs, respectively, thus encompassing sequences specific for RNAs 5 and 6, respectively (Fig. 7A). In contrast, three other cDNAs (4-11, 4-6, and 4-35), all of which carry continuous ORFs, hybridize only to the genomic RNA (12–13 kb). cDNA 4-37 also hybridizes to all LDV RNAs (Fig. 7A), in spite of the fact that it exhibits no homologies to any of the other cDNAs. A restriction fragment of the 5' end of cDNA 4-37 also hybridizes to all subgenomic RNAs, whereas the 3'-end restriction fragment hybridizes only to the genomic RNA (L. Kuo *et al.*, unpublished data). On the basis of the genome organization of EAV (see later, Section III, E), the results indicate cDNA 4-37 is located close to the 5' end of the genome and that its 5' end is part of a common leader sequence that is attached to all subgenomic RNAs. This conclusion is supported by combined reverse transcription/polymerase chain reaction (PCR) analyses using antisense oligonucleotide primers to the 5' termini of the three ORFs at the 3' end and a sense oligonucleotide primer to the 5' end of cDNA 4-37 (Z. Chen *et al.*, unpublished data 1991). Specific 200- to 300-bp PCR products were generated that identified a common junction between the leader sequence and the bodies of the subgenomic RNAs, 5'-UAACC-3', which is similar, but not identical, to the junction sequence identified for EAV 5'-UCAAC-3' (see Section III,E). More than a common leader sequence and junction segment, however, must be involved in the regulation of the synthesis of individual subgenomic RNAs, because they are consistently produced in different amounts (Fig. 7A). RNAs 8 and 6 always seem to be produced in highest amounts, especially RNA 8. This arrangement makes sense considering the efficiency of replication of viruses in general, because RNA 8 encodes the nucleocapsid protein, VP-1, and RNA 6 may encode the envelope glycoprotein, VP-3.

cDNA 4-11 (1620 nucleotides) encodes an ORF with a replicase motif typically found in positive-strand RNA viruses (Argos, 1988; Gorbalenya *et al.*, 1989) (Fig. 8). The only abnormality is that the GDD triplet characteristic for this motif (Argos, 1988) is a SDD triplet in the LDV replicase motif, but the LDV motif shares this SDD triplet with

CONSENSUS AMINO ACIDS	D D		SG T N		GDD
FLAVIRUSES (NS-5)	YAD*TAGW*TRITEADL KVLNDLK RT	47/48	RRDQRG**QVV*YAL*TITN KE G G F E L	42-45	MAVS***CVVR LI K
PESTIVIRUSES (p133)	VSF*TKAW*TQVTSKLQ L Y TRDL	39/40	RNGQRG**QPD*SAG*SMLN	24	IHVC***GFLI
HepCV ("NS-5")	FSY*TRCF*STVTESDI N	42	YRRCRA**VLT*SCG*TLTC	17	MLVC***LVVI N
ALPHAVIRUSES (NSP4)	LET*IASF*KSQDDAMA SL	39	FGAMMK**MFL*LFV*TVLN I	17	AAFI***NIIH V
RUBIVIRUS (NSP4)	IEV*FTEF*MNQTLATR	37	TGCERT**EPA*LLH*TTVA	15	GIFQ***MVIF
EAV	LGT*LESC*RSTPALVR	37	KRGGLS**DPI*SIS*TIYS	39	VYIYS**VVLT
LDV-P	LEA*LASC*RSTPAIIR	39	KRGGLS**DPV*SIS*TVYS	38	LLVYS**VVFY
IBV (ORF 1b)	MGW*YPKC*RAMPNILR	44	KPGGTS**DAT*AYA*SVFN	59	LMILS**GVVC
MHV-A59 (ORF 1b) -JHM	MGW*YPKC*RAMPNILR	44	KPGGTS**DAT*AFA*SVFN	59	MMILS**GVVC I
BEV (ORF 1b)	FGS*YTKC*RTFPLSFR	40	KPGGTS**DAT*AHT*TFYN	52	LNFLS**SFIF

FIG. 8. Summary of replicase motifs for flaviviruses, pestiviruses, hepatitis C virus, alphaviruses, rubivirus, EAV, LDV, IBV, MHV, and BEV. The summary includes the replicase motifs of the following flaviviruses: yellow fever virus (Rice *et al.*, 1985), Dengue virus 2 (Hahn *et al.*, 1988) and 4 (Mackow *et al.*, 1987), Kunyan virus (Coia *et al.*, 1988), and tick-borne encephalitis virus (Mandel *et al.*, 1989); of the following pestiviruses: bovine viral diarrhea virus (Collett *et al.*, 1988) and hog cholera virus (Meyers *et al.*, 1989); of rubivirus (Dominguez *et al.*, 1990); of hepatitic C virus (HepCV)) (Choo *et al.*, 1989; Kato *et al.*, 1990); of the following alphaviruses: Sindbis virus (Strauss *et al.*, 1984) and Semliki Forest virus (Takkinen, 1986); of LDV (Kuo *et al.*, 1991), of EAV and MHV-A59 (Bredenbeck *et al.*, 1990); of MHV-JHM (Lee *et al.*, 1991); of IBV (Boursnell *et al.*, 1987); and of Berne virus (BEV) (Snijder *et al.*, 1990).

those of the 1b proteins of EAV; the coronaviruses, infectious bronchitis virus of chicken (IBV) and MHV; and the torovirus, Berne virus (BEV; see Fig. 8). The replicase motif sequences of the EAV and LDV genomes are highly homologous; 36 of the total 48 amino acids shown in Fig. 8 (75%) are identical. Furthermore, the replicase motifs of EAV and LDV resemble to a greater extent those of the two coronaviruses and BEV (44% amino acid identity) than those of the flaviviruses, pestiviruses, and alpha and rubi togaviruses. The protein encoded by cDNA 4-11 also contains a zinc finger motif toward its carboxyl end, similarly to the 1b proteins of EAV and coronaviruses. Another protein with a cysteine-rich segment is encoded by cDNA 4-35 (1259 nucleotides). On the basis of the genome organizations of EAV (den Boon *et al.*, 1991) and of the coronaviruses and toroviruses (Spaan *et al.*, 1990), it seems likely that cDNAs 4-11, 4-6, and 4-35 represent sequences of an 8- to 9-kb replicase gene located at the 5' end of the LDV genome (for comparison to the EAV genome, see Section III,E). Combined, the results discussed above

suggest the tentative genome organization of LDV-P illustrated in Fig. 7B. The exact location of cDNAs 4-35, 4-11, and 4-6 is uncertain. Their relative placement in Fig. 7B is suggested on the basis of some degree of homology between short segments of these cDNAs and sequences in the EAV genome (cross-hatched bars).

3. *LDV Variants*

LDV has been isolated from numerous transplantable tumors carried in different strains of mice (Rowson and Mahy, 1975; 1985). Six isolates commonly studied and some of their properties are summarized in Table IV. The isolates differ serologically, in neurovirulence, and in RNA sequence. The LDV contaminant of Ib leukemia cells, which had been passaged numerous times over the years through C58 mice, and

TABLE IV

DIFFERENCES IN NEUROVIRULENCE AND IMMUNOLOGICAL SPECIFICITY IN DIFFERENT ISOLATES OF LDV

LDV isolates		Incidence of paralysis, % of total C58 mice injected at age (months)[b]			Neutralization by rabbit anti-LDV prepared to LDV-P[c]
Designation origin[a]	Refs.[a]	6	9	12	
Ib-LDV,LDV-C, LDP-M, LDV-v	1–4	>90	>90	>90	+
LDV-P	5	0	33	81	+++
LDV-N	6	10	60	60	+
LDV-S	7	0	ND[d]	90	+
LDV-RIL	8	0	38	32	+
LDV-a	9	0	0	0	ND

[a] Note: references pertain to origin of LDV isolates.

(1) Nawrocki *et al.* (1980); (2) Martinez *et al.* (1980); (3) Contag *et al.* (1986a); (4) Contag and Plagemann (1989); LDV-M and LDV-v are derivatives of Ib-LDV, which, like LDV-C, has been isolated from C58 mice injected with Ib leukemia cells that apparently were contaminated with LDV; (5) Darnell and Plagemann (1972); (6) Notkins and Shochat (1963); (7) Michaelides and Schlesinger (1973); (8) Riley *et al.* (1960); (9) LDV-a (avirulent) is a derivative of W-LDV (Murphy *et al.*, 1983). The origin of the latter has not been described.

[b] The data are compiled from Martinez *et al.* (1980), Nawrocki *et al.* (1980), and Murphy *et al.* (1983). Groups of 9–16 C58 mice of the indicated ages were immunosuppressed by X-irradiation or cyclophosphamide treatment 1 day before infection with the indicated isolate of LDV.

[c] Data are from Cafruny and Plagemann (1982b). +++, About 99% neutralization in 10 min of incubation at 4°C with serum from a rabbit immunized with LDV-P; +, <90% neutralization in 60 min of incubation at 4°C with the same antiserum.

[d] ND, Not determined.

its derivatives (LDV-C, LDV-M, and LDV-v) exhibit the greatest neurovirulence (Table IV). In contrast, another LDV isolate, LDV-a, was found not to cause any paralytic disease regardless of the age of the C58 mice, when the mice were immunosuppressed by either a single dose of X-irradiation 1 day before LDV infection (Table IV) (Murphy *et al.*, 1983) or multiple injections of cyclophosphamide (G. W. Anderson and P. G. W. Plagemann, unpublished observations 1991). Five other LDV isolates showed intermediate neurovirulence when assayed under the same conditions, but their apparent neurovirulence seemed to increase with increase in age of the test mice (Table IV). This dependence of neurovirulence on the age of the test mice, combined with the realization of the importance of an anti-LDV immune response in ADPM resistance (see Table II), raises the possibility that the apparent differences in neurovirulence of some of the various LDV isolates may not be related to differences in their ability to infect or replicate in susceptible motor neurons, but perhaps to a higher immunogenicity in C58 mice relative to that of the Ib-LDV that had been passaged continuously in C58 mice. If this view is correct, differences in apparent neurovirulence may disappear if the test C58 mice are repeatedly injected with cyclophosphamide to achieve a prolonged inhibition of an anti-LDV immune response (see Table II). Experimental evidence available at present does not address this possibility except in the case of LDV-a, which does not induce ADPM even if the mice are continuously immunosuppressed (see above). However, upon 10 5-day passages in 12-month-old C58 mice, LDV-a showed a progressive increase in neurovirulence for immunosuppressed 10-month-old C58 mice (Murphy *et al.*, 1983). This change could reflect selection of a less immunogenic variant by passage in old C58 mice or selection of LDV variants that replicate with greater efficiency in motor neurons, a selection comparable to the cell type-specific selection of variants of LCMV in mice (Ahmed and Oldstone, 1988) or of HIV in humans (Fauci, 1988).

In contrast, a similar repeated passage of the neurovirulent Ib-LDV in BALB/c mice resulted in loss of neurovirulence (Murphy *et al.*, 1983). The latter finding has been confirmed (G. W. Anderson *et al.*, unpublished data 1991); LDV-v had lost all its neurovirulence for 8-month-old C58/M mice after one 21-day passage in BALB/c mice whether the C58/M mice received single or multiple treatments of cyclophosphamide 1 day before LDV infection. Interestingly, when LDV-v was passaged in BALB/c mice by harvesting the virus in each passage 1 day postinfection, no loss in neurovirulence occurred during six passages. These results indicate that the selective pressure for the isolation of a neurovirulent variant in C58 mice and of an avirulent variant in

BALB/c mice develops with time after infection. The nature of the selective pressure, which could occur at the level of neuron or macrophage infection or involve an immunological component, has not been elucidated.

Some kind of mouse strain-specific immunological pressure could also explain antigenic differences between various LDV isolates, since these have been isolated from transplantable tumors that have been generally carried in different mouse strains. The antigenic differences between LDV isolates was apparent only at the level of virus neutralization (Table IV). Polyvalent anti-LDV antibodies prepared by immunizing a rabbit with LDV-P efficiently neutralized the infectivity of LDV-P, but only very inefficiently neutralized the infectivity of four other LDV isolates. The rabbit antiserum partially neutralized another LDV isolate, LDV-ROW (Cafruny and Plagemann, 1982b). Interestingly, the LDV isolated from *M. caroli* after infection with LDV-ROW, LDV-RMC (Rowson, 1980), was neutralized as efficiently as LDV-P by the rabbit anti-LDV-P serum (Cafruny and Plagemann, 1982b). Thus antigenic variants, like neurovirulent variants, become rapidly selected and the selective pressure seems mouse strain specific. Although at least two serological types of LDV are recognized by neutralization with rabbit anti-LDV antibodies, polyclonal mouse antibodies to all isolates of LDV reacted similarly with LDV-P in an ELISA (Cafruny and Plagemann, 1982b), and macrophages infected with the various LDV isolates likewise reacted about equally with polyclonal mouse anti-LDV-P and anti-LDV-M antibodies in an indirect fluorescent antibody staining assay (B. Hu and P. G. W. Plagemann, unpublished observations). Furthermore, VP-3 of LDV-M and LDV-P cross-reacted in Western blots using anti-LDV antibodies to the two LDV isolates regardless of whether they were propagated in BALB/c, Swiss, or C58 mice, and polyclonal mouse antibodies to both LDV-P and LDV-M efficiently protect susceptible motor neurons from infection by LDV-M (Harty *et al.*, 1987a,b). In addition, LDV-P and LDV-M are about equally neutralized by the neutralizing MAbs raised to formalin-inactivated LDV-P (Harty and Plagemann, 1988), even though they can be distinguished by polyclonal neutralizing rabbit anti-LDV-P antibodies (see Table IV). Thus the epitope recognized by the neutralizing MAbs to formalin-inactivated LDV-P is more broadly specific than the distinct epitope(s) recognized by the neutralizing rabbit anti-LDV-P antibodies. The recognition of distinct neutralizing epitopes by the neutralizing anti-LDV-P mouse MAbs and polyclonal rabbit antibodies is also indicated by the isolation of a neutralization escape variant that is no longer neutralized by the former, but is neutralized by the latter as

well as by polyclonal anti-LDV antibodies that arise during a natural infection (Harty and Plagemann, 1988). LDV isolates also seem to differ in their ability to induce autoantibody formation in mice. Infection of STU mice with LDV-P and LDV-ROW resulted in a lower formation of anti-Golgi apparatus autoantibodies than infection with LDV isolated from STU mice carrying an LDV-contaminated transplantable tumor (Weiland *et al.*, 1987).

The molecular basis of the differences in neutralizing epitopes and neurovirulence among LDV isolates has been explored only to a limited extent. One technical difficulty is that no cloning procedure is presently available for LDV. One substitute approach has been to isolate LDV from mice injected with the highest dilution of virus inoculum that resulted in infection in an end-point dilution assay and then to amplify the virus by a 1-day passage through another group of mice. By T1 RNase fingerprinting, 2 or 3 of 30 unique oligonucleotides of LDV-P and LDV-M RNA were found to be distinct (Contag *et al.*, 1986b), and up to 13 distinct oligonucleotides have been detected in the RNAs of LDP-P, LDV-C, LDV-RIL, LDV-N, and LDV-S (Brinton *et al.*, 1986a). The RNA of each isolate yielding a unique T1 fingerprint. Interestingly, the RNAs of two derivatives of LDV-RIL yielded distinct T1 fingerprints and were estimated to differ from each other by 1.5% (Brinton *et al.*, 1986a). In addition, RNA T1 fingerprints of LDV-C changed significantly during two 1-day passages in C58 mice (Brinton *et al.*, 1986a). Results from direct sequencing of approximately 100 nucleotides in the 3'-end region of the genome indicated that LDV-C differed from LDV-N, LDV-S, and one derivative of LDV-RIL by 11, 5, and 13 nucleotides, respectively, and that the LDV-RIL RNA contained two deletions (Godeny *et al.*, 1989). Three nucleotide differences were common to LDV-N, LDV-S, and LDV-R. Sequence analysis has shown that the 3'-terminal noncoding segments of 80 nucleotides of LDV-C and LDV-P are identical but that overall, the 3' terminal 1064 nucleotides of LDV-C and LDV-P exhibit only about 88% identity (Kuo *et al.*, 1991; L. Kuo *et al.*, unpublished observations 1991). Most of the nucleotide differences are silent because the ORFs for VP-1 (115 amino acids) and VP-2 (171 amino acids) of the two variants differ only by 2 (1.3%) and 7 (4.6%) amino acids, respectively. Overall, these results further support the view that LDV is subject to rapid genetic changes and that multiple genetic variants can be generated that are infectious for macrophages from different mouse strains. To what extent these variants become selected under specific environmental conditions, such as in different inbred mouse strains or during the persistent infection of a mouse of a certain strain, and what selective pressures are operating

are interesting but unanswered question. These questions will be more readily approachable once an LDV genome has been completely sequenced and its gene organization elucidated.

D. Transmission and Persistence in Nature

LDV has been isolated from wild mice in various parts of the world, but clearly not all wild mice in nature are infected. (Rowson and Mahy, 1975, 1985; Brinton, 1986). The question is why not, because infected mice retain a relatively high viremia for life, LDV is secreted in feces, urine, and saliva, and LDV is highly infectious when injected, regardless of the route of injection (Rowson and Mahy, 1975, 1985). In fact, the particle/mouse infectivity ratio of injected LDV is very low and probably approaches one. Mice in nature probably escape infection due to a low rate of transmission. LDV is transmitted from mother to offspring, most likely transplacentally, and perhaps through milk, but the incidence of transmission seems low, especially from persistently infected mothers (Rowson and Mahy, 1975, 1985). Horizontal transmission seems to be limited by the mucosal barrier because the infection of mice by LDV via vaginal, rectal, and oral routes required LDV doses four to six orders of magnitude higher than for intraperitoneal injection (Cafruny and Hovinen, 1988b). However, infection of mice via the oral route can be enhanced by nonsteriodal antiinflammatory agents (Cafruny *et al.*, 1991). Mucosal barriers can be broken by bloodsucking parasites as is the case in the transmission of many blood-borne viral infections. This is a potential route of transmission of LDV in nature, but no parasitic vector for LDV has been identified. Transmission is commonly observed between male laboratory mice that are fighting and biting. On the other hand, transmission between female laboratory mice housed together is rather infrequent (Rowson and Mahy, 1975, 1985).

Nevertheless, at the time of the discovery of LDV, some colonies of laboratory mice were infected with LDV (Plagemann *et al.*, 1963). This fact may explain why many transplantable mouse tumors and murine viruses that were maintained by mouse passage became LDV carriers (Rowson and Mahy, 1975, 1985; Riley *et al.*, 1978; Horzinek, 1981). Once inadvertently passed through an LDV-infected mouse, tumor cells or infectious agents will remain contaminated with LDV as long as they are maintained by mouse passage, because of the relatively high blood levels of LDV in infected mice. As summarized previously (Riley, 1974; Riley *et al.*, 1978; Brinton, 1986), the presence of LDV as a contaminant has compromised results from many studies in which various host responses to LDV infection were erroneously attributed to

implanted tumors, other viruses, or certain experimental factors. Thus it seems prudent to consider the potential presence of LDV in various types of mouse experiments. An LDV infection of a mouse can be readily detected by a 5- to 10-fold elevation of plasma LDH activity. Contaminated tumors can be freed of LDV by propagation in culture for 2–3 weeks or passage through another species. Likewise, other viruses can be freed of LDV by passage through another species or by propagation in cultures of cells not containing permissive mouse macrophages (Rowson and Mahy, 1975, 1985; Brinton, 1986).

III. Equine Arteritis Virus

EAV was first isolated from lung tissue of an aborted fetus during the first recognized outbreak of equine arteritis among horses on a farm in Bucyrus, Ohio in 1953 (Doll *et al.*, 1957a). Aspects related to the disease have previously been reviewed by Mumford (1985), van Berlo (1985), and Kaaden *et al.*, (1990), and aspects related to the morphology, genome organization, and replication of EAV by Brinton (1980), Horzinek (1981), and Spaan *et al.*, (1990).

A. Pathogenesis

The first recognized outbreak of equine arteritis in Bucyrus was one of the most severe on record. It was introduced into a stud wherein the majority of mares were pregnant and resulted in abortion in 30 of 60 mares (Doll *et al.*, 1957b). Infrequent epizootics have subsequently been reported from various parts of the United States and Europe (Mumford, 1985; Kaaden et al., 1990). Serological evidence, however, indicates that infections have been much more widespread than indicated by these outbreaks. A recent serological survey in Germany showed that, although no clinical cases of equine arteritis have been reported in that country, 28 of 739 horse serum samples examined (3.8%) had significant anti-EAV antibody titers (Kaaden *et al.*, 1990). This represented more than a doubling of the percentage of antibody-positive sera between 1987 and 1989. A considerable proportion of sero-positive horses have also been reported from various surveys between 1975 and 1989 in France, Argentina, Canada, Morocco, and the United States (Kaaden, *et al.*, 1990). Most often infections, even in epizootics, seem to be subclinical or to involve mild, often unrecognized, infections of the respiratory tract resembling influenza (Jones, 1969; Mumford, 1985).

Clinical symptoms have been found to vary widely. Typical signs include pyrexia for 1–5 days, anorexia and depression, lacrimation,

conjunctivitis, leg edema, serous nasal discharge, and congestion of the nasal mucosa (Mumford, 1985; Kaaden *et al.*, 1990). Clinical disease is generally more severe among horses experimentally infected with the virulent Bucyrus strain than those with natural infections, often resulting in death. The reasons for the differences in morbidity and mortality have not been resolved. Most likely they reflect, at least in part, the prevalence of EAV variants differing in virulence. Indeed, a strain isolated from the spleen of a naturally infected horse in a relatively mild epizootic (Penn) caused a milder disease than the Bucyrus strain in experimental infections of horses (McCollum *et al.*, 1961). There is also the suggestion that variants exist that differ in their abortogenic potential (Doll *et al.*, 1957a; Cole *et al.*, 1986; Golnick *et al.*, 1986). However, other factors, such as differences in susceptibility of various types of horses to EAV infection (Mumford, 1985), may also play a role. It is also unclear how the virus is maintained between outbreaks of equine arteritis and what factors are responsible for the development of epizootics. Perhaps the generation and selection of variants with increased virulence play a role. Though EAV infections seem to be widespread in endemic areas, no infections have been observed in Great Britain, Japan, and recently in the Netherlands; serological surveys in these countries have been negative, except for positive sera from imported horses (Mumford, 1985; van Berlo, 1985).

Pathological lesions may consist of edema, congestion, and hemorrhage of subcutaneous tissues, lymph nodes, and viscera of the peritoneal and pleural cavities (Doll *et al.*, 1957a). In terminal stages of experimentally infected horses, widespread necrotizing arteritis affecting the media of the small muscle arteries has been observed. This finding has been the basis of the name of the disease, equine arteritis (Jones *et al.*, 1957; Estes and Cheville, 1970).

Studies of horses after experimental intranasal infection with the Bucyrus strain of EAV indicated that initial viral replication occurs in macrophages of the lung (McCollum *et al.*, 1971; Crawford and Henson, 1973). Viral antigen has been found in macrophages around bronchioles (Crawford and Henson, 1973). The virus then spreads to bronchial lymph nodes by the second day postinfection and from there seems to become disseminated throughout the body via the circulatory system. Dissemination could be via free virions or infected macrophages. Viral antigen can be detected in practically all tissues, except the brain. Macrophages, present throughout all tissues, might be the primary cell supporting EAV replication in the animal (Mumford, 1985). Secondary sites of replication have been identified as medial and endothelial cells. The question of whether vascular lesions and arterial damage are mediated through EAV replication in macrophages or involve infection

of other types of cells or various indirect effects does not seem to have been resolved. Neither has the cause of abortions associated with infections of pregnant mares been elucidated. It has been suggested to result from lesions in the uterus (Coignoul and Cheville, 1984).

Another unanswered question concerns the possibility of persistent infections of horses. EAV has been isolated from the buffy coat and lung from an apparently healthy horse (Fukunaga *et al.*, 1981). Also, although lesions in the vascular and lymphatic systems were found to have largely subsided 10 days postinfection in horses that had clinically recovered from the infection, arterial damage persisted for several weeks (Crawford and Henson, 1971). In addition, it has been reported that renal lesions can persist for long periods of time (Prickett *et al.*, 1973) and that EAV could be isolated from renal tissues of experimentally infected horses long after it seemed to have disappeared from other tissues (McCollum *et al.*, 1971). It seems unclear whether persistent renal lesions are caused by EAV replication in tissue cells per se or are the result of the accumulation of complement-fixing antibody–virus complexes. A more definitive chronic carrier state has been identified in naturally infected thoroughbred stallions (Timoney *et al.*, 1986, 1987). Carrier stallions shed infectious EAV in semen for at least 1–2 years. Although the combined data suggest that low-level EAV infections may persist in horses, little is known about the efficiency with which EAV can establish such persistent infections and how the virus evades host defense mechanisms (see later). The frequency of a long-term carrier state varied considerably between groups of stallions on different farms (Timoney *et al.*, 1986).

The primary mode of transmission has been suggested to be by aerosol via the respiratory route. Although EAV clearly is transmitted between horses, little is known about the efficiency of the transmission under natural conditions. Veneral transmission is likely because EAV has been isolated from semen (Timoney *et al.*, 1986). In fact, it has been suggested that carrier stallions might play an important epidemiological role in the dissemination and perpetuation of EAV (Timoney *et al.*, 1986). Infection of mares via artificial insemination with contaminated semen has been documented. More information is needed on the potential long-term dissemination of the virus via urine excreted by persistently infected horses with evidence of kidney infection (Mumford, 1985). One complicating factor in transmission studies has been that it has proved difficult to isolate EAV from naturally infected horses even during the acute febrile phase of the infection (Mumford, 1985). This problem might become tractable by application of modern techniques of molecular biology. Indeed, a molecular test has recently been developed for the amplification of EAV RNA and applied to its detection in semen

(Chirnside and Spaan, 1990). The RNA was reversed transcribed using negative sense oligonucleotides to the 5′ leader, the 3′ nucleocapsid gene, and the replicase gene of the viral genome (see later) as primers, and the transcription products were amplified by PCR after addition of appropriate positive oligonucleotide primers.

Isolation of EAV from horses generally consists of inoculating cultures of susceptible cells (see below) with nasopharyngeal, vaginal, and rectal swabs, serum, a buffy coat fraction from blood, or semen (Doll *et al.*, 1968; McCollum *et al.*, 1971; Fukunaga *et al.*, 1981; Kaaden *et al.*, 1990). Infection is generally recognized by the development of cytopathic effects within 6–10 days after inoculation. Serial blind passage in cell culture has also been employed to amplify infectious virus (Mumford, 1985). The PCR method for detecting EAV RNA promises to be much more sensitive, rapid, and in the long run cost effective for detecting EAV in semen, tissues, and body cavity swabs once the PCR method becomes more widely used as a diagnostic test. An added advantage is that sterility is not a requirement.

B. Replication in Cell Culture and Virus Growth Cycle

EAV was first shown to replicate in primary cultures of kidney cells from horses (McCollum *et al.*, 1961), rabbits (McCollum *et al.*, 1962), and hamsters (Wilson *et al.*, 1962). However, EAV also efficiently replicates in a variety of cell lines: baby hamster kidney cells (BHK21) (Hyllseth, 1969), rabbit kidney cells (RK-13, LLC-RK1) (McCollum, 1976; Radwan and Burger, 1973), African green monkey cells (Vero, B-SC-1) (Hyllseth, 1973; Maes *et al.*, 1970; Crawford and Henson, 1973), rhesus monkey cells (LLC-MK2) (McCollum *et al.*, 1971), and a diploid line of equine dermal origin (NBL-6) (Breese and McCollum, 1971; McCollum, 1986). EAV replication in these types of cells is generally cytocidal, which allowed the establishment of tissue culture endpoint dilution assays (Wilson *et al.*, 1962) and of plaque assays in BHK21, RK-13, or Vero cells for its quantitation (Hyllseth 1969; Maes *et al.*, 1970; van Berlo *et al.*, 1980; Fukunaga *et al.*, 1981). Other susceptible cell lines have also been found satisfactory for plaque assays of EAV (Horzinek, 1981). DEAE–dextran has been found to enhance plaque formation (Horzinek, 1981).

Viral RNA synthesis in BHK21 and Vero cells as measured by the incorporation of [^{3}H]uridine in the presence of actinomycin D is first detectable about 4 hr postinfection with the Bucyrus strain of EAV (van Berlo *et al.*, 1982) (Fig. 9). Autoradiographic analysis of [^{3}H]uridine-labeled, infected cultures and immunocytochemical staining for viral

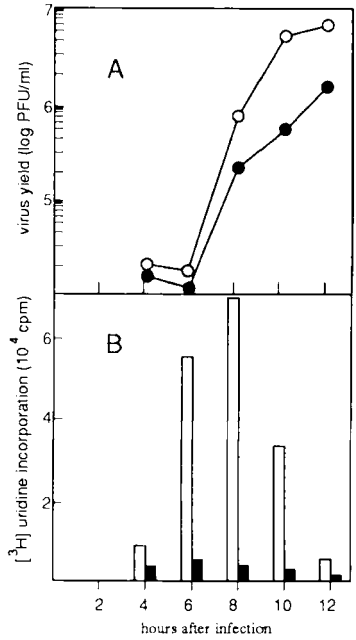

FIG. 9. Kinetics of virus growth (A) and viral RNA synthesis (B) in EAV-infected BHK21 cells. Cells were infected with 30 pfu/cell and incubated in the absence of actinomycin D (○) or with this drug added 2 hr postinfection (●). In the latter case the kinetics of the synthesis of viral RNA were also measured by pulse labeling actinomycin-treated, infected cultures for 1 hr with [^{3}H]uridine at 2-hr intervals. Open bars, infected; filled bars, mock infected. (Data from van Berlo *et al.*, 1982; reproduced with permission of the authors and publisher.)

antigens indicated that practically all cells in these cultures became productively infected (van Berlo *et al.*, 1980). These results contrast with the earlier conclusion, derived from infectious center assys, that only a limited number of cells become infected in these cultures (Horzinek, 1981). Maximum rates of viral RNA and protein synthesis are observed in one-step growth experiments at 35–37°C between 6 and 8 hr postinfection and virus release begins about 6–8 hr postinfection and is complete by 10–20 hr postinfection depending on the incubation temperature (Maes *et al.*, 1970; van Berlo *et al.*, 1982, 1986b; van der Zeijst *et al.*, 1975) (Fig. 9A). Maximal viral yields of between 10^7 and 10^8 plaque-forming units (pfu)/ml of culture fluid have been obtained regardless of the incubation temperature (35, 37, or 40°C) (Horzinek, 1981). Immunofluorescent antibody staining indicated that EAV anti-

gens first appear as small granules in the perinuclear region and then spread throughout the cytoplasm (Breese and McCollum, 1971). Electron microscopic studies indicated that the virus matures by budding into intracytoplasmic cisternae (Magnussen *et al.*, 1970) (Fig. 10A). Budding from the cell surface was not observed (Magnussen *et al.*, 1970). Cytopathic effects of EAV replication are characterized by rounding of the cells and detachment from the culture plate surface and become apparent 10–12 hr postinfection (van Berlo *et al.*, 1980, 1982).

C. Host Immune Response and Vaccination

Most serological surveys for anti-EAV antibodies in horses (Mumford, 1985) have been based on on the detection of neutralizing anti-EAV antibodies as measured by a plaque reduction assay (Hyllseth, 1969; Hyllseth and Petterson, 1970; Radwan and Crawford, 1974; Fukunaga *et al.*, 1981) or by measuring the reduction of tissue culture

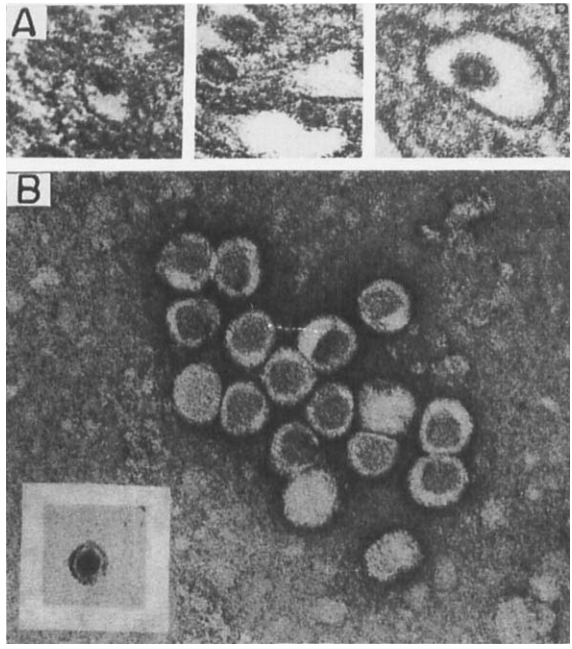

FIG. 10. Electro micrographs of a section of EAV-infected BHK21 cells (A) and of EAV purified from the cultured fluid (B). [Data from Magnusson *et al.* (1970) and Maess *et al.* (1970), respectively.] Magnification, (in A): × 161,400. The mean diameter of the virions in B is 60 nm. (Reproduced with permission of the authors and publishers.)

infectious doses in the presence of complement using a variety of cell culture lines (Wilson *et al.*, 1962; McCollum, 1986; Cook *et al.*, 1989). Kits based on the latter procedure are commercially available. Complement fixation techniques have been developed for the detection of anti-EAV antibodies (Crawford and Henson, 1973; Fukunaga and McCollum, 1977) but have not found wide application. Recently, an ELISA has been established for this purpose using purified cell culture-propagated EAV (Lang and Mitchell, 1984). Although an ELISA has the advantage of being less restricted in antibody detection and more rapid than the neutralization assay and is as sensitive as the latter it has been found to suffer from false-positive results in some instances (Cook *et al.*, 1989). These arise, on the one hand, from difficulties in freeing purified EAV of bovine serum proteins and, on the other hand, from the presence of antibodies to bovine serum in horses that have been vaccinated with other cell culture-derived viruses, such as equine herpes virus (Cook *et al.*, 1989).

Neutralizing anti-EAV antibodies appear in horses within a week after natural or artificial infection (Doll *et al.*, 1968; Fukunaga and McCollum, 1977; Fukunaga *et al.*, 1981). The appearance of neutralizing antibodies was found to be associated with the disappearance of infectious virus from the serum of the infected horses, though virus could still be isolated from the buffy coat and lung for 36 days postinfection (Fukunaga *et al.*, 1981). Neutralizing antibodies are predominantly IgG; little or no neutralizing anti-EAV IgM has been detected, except during early times postinfection (Hyllseth and Petterson, 1970; Radwan and Crawford, 1974). IgG antibodies neutralize EAV infectivity for cultured cells relatively inefficiently, but their effectiveness, especially when obtained at late times postinfection, is greatly enhanced by the presence of complement (Hyllseth and Petterson, 1970; Radman and Crawford, 1974; Radwan *et al.*, 1973; Radwan and Burger, 1973). Complement causes lysis of the virions with release of the viral; RNA (Radwan and Crawford, 1974). Neutralization of EAV by anti-EAV antibodies generated in a variety of other species is also greatly enhanced by complement (Hyllseth and Petterson, 1970; Radwan and Crawford, 1974; Radwan *et al.*, 1973). Thus this effect of complement is related to a property of the virions, rather than to a property of horse IgG. High titers of neutralizing antibodies may persist for at least 7 years (Mumford,, 1985). It became apparent early after the initial discovery of EAV that the presence of serum-neutralizing anti-EAV antibodies acquired by either natural or artificial infection protects horses against a challenge infection by the virulent Bucyrus strain of EAV (McCollum, 1969). However, protection has generally been evalu-

ated as prevention of clinical disease after a challenge infection (McCollum, 1969, 1986). It has become apparent that the presence of neutralizing anti-EAV antibodies does not completely protect horses from reinfection, only against clinical disease (see later).

The recognition of the protective effect of anti-EAV antibodies and the discovery that EAV can be propagated in cell culture triggered attempts to develop an attenuated vaccine (McCollum *et al.*., 1961; Doll *et al.*, 1968; McCollum, 1969, 1976, 1981, 1986). The Bucyrus strain was first passaged repeatedly through cultures of horse kidney cells and later through cultures of rabbit kidney cells and equine epidermal cells. Early studies indicated that cell culture passage reduced the virulence of the virus for horses, that intramuscular vaccination with cell culture-passaged EAV protected against challenge infection, and that the virus was not transmitted from the challenged vaccinated horses to in-contact, susceptible animals (McCollum *et al.*, 1961). Additional cell passages further reduced the virulence of the EAV. The latest attenuated strains have been passaged 131 times in horse kidney cell cultures, 110 times in rabbit kidney cell cultures, and 19 and 25 times in equine dermal cell cultures (HK131-RK110-AK19 or 25) (McCollum, 1986). Administration of these strains did not induce any clinical signs, but induced neutralizing anti-EAV antibody responses in all of 19 vaccinated horses and conferred complete protection from clinical disease in 14 of them in response to a challenge infection (McCollum, 1986). Comparable results were reported from another study (Fukunaga *et al.*, 1984). The infectivity and immunizing potency of the attenuated live virus vaccine (as a lyophilized suspension of virus in a medium containing 2–5% fetal bovine or horse serum) have been found to be completely stable at −20°C for a year and relatively stable at 4°C, but rapidly lost stability at 23–28°C and 37°C (Harry and McCollum, 1981). One complicating factor of the live attenuated vaccines, however, is that exposure of vaccinated horses to wild-type virus generally results in reinfection and transient secretion of virus (McCollum, 1981, 1986; Mumford, 1985). Killed or subunit virus vaccines would avoid this complication, and first attempts to develop a killed vaccine have been reported (Fukunaga *et al.*, 1984). Two sequential injections of cell culture-propagated and partially purified EAV that had been inactivated by treatment with formalin induced serum-neutralizing antibody titers higher than those observed in horses vaccinated with the live virus vaccine.

Vaccination with attenuated EAV seems to be limited to certain regions in the United States and no further application of the killed virus vaccine has been reported. A live vaccine is commercially avail-

able (Arvac, Fort Dodge Laboratories). To prevent further spread of EAV in Germany, Kaaden *et al.*, (1990) have proposed a voluntary prophylactic program that consists of serological tests of studs for anti-EAV antibodies, examination of semen of seropositive studs for EAV, and exclusion of secretors from breeding. In addition, it is suggested to restrict the import of seropositive mares and studs, and sperm of the latter, as is already practiced in some countries (Kaaden *et al.*, 1990).

D. Viral Strains

The epidemiology of equine arteritis suggests the prevalence of strains that differ in virulence for horses, but concrete evidence on this point is not available, except for the finding mentioned already, that the Penn isolate seems less virulent than the Bucyrus isolate. To what extent antigenic variants exist is equally uncertain. Vaccination of horses with isolates from six different epizootics (five from the United States and one from Europe) about equally protected them from challenge infection by the virulent Bucyrus strain (McCollum, 1969). This finding suggests the prevalence of a single serotype with respect to the immune protection of horses. However, limited *in vitro* cross-neutralization assays have suggested some antigenic variation among different isolates (Fukunaga and McCollum, 1977).

E. Virion Properties and Genome Organization

EAV is an enveloped virus with a diameter of 60 ± 13 nm and a nucleocapsid core with a diameter of about 35 nm (Fig. 10B and Table I) (Maess *et al.*, 1970; Estes and Cheville, 1970; Horzinek *et al.*, 1971; Horzinek, 1981). The virions are spherical and lack prominent surface projections. The surface appears to be composed of 12- to 14-nm-wide ringlike structures similar to those observed in LDV envelopes (Horzinek *et al.*, 1971; Horzinek, 1981). The buoyant density of EAV in sucrose density gradients has been reported as 1.17 g/cm^3 (Hyllseth, 1970), 1.2 g/cm^3 (Maess et al., 1970), or 1.16 g/cm^3 (van der Zeijst *et al.*, 1975), and its sedimentation coefficient as 224 ± 8 S (van der Zeijst *et al.*, 1975). EAV has not been found to agglutinate red blood cells from any species investigated (Horzinek, 1981).

The core nucleocapsid exhibits a sedimentation coefficient of 158 S (Zeegers *et al.*, 1976). It is composed of the 48 S (~13 kb) genomic positive-strand RNA (van der Zeijst *et al.*, 1975; Hyllseth, 1973; Zeegers *et al.*, 1976; van Berlo *et al.*, 1986a) and the phosphorylated nucleocapsid protein VP-1 of 12–14 kDa. VP-1 binds directly to staphylococcal

protein A (van Berlo *et al.*, 1983). A nonglycosylated protein, VP-2, of 17–18 kDa, is associated with the envelope (Zeegers *et al.*, 1976; van Berlo *et al.*, 1986a,b). The number of glycoproteins and their molecular weights are not completely defined. The first study concerning the structural proteins of EAV (Hyllseth, 1973) identified approximately six glycosylated proteins ranging from 28 kDa to 72 kDa in EAV purified from cultures of infected BHK21 cells. A later comparison of the proteins of EAV purified from cultures of three different types of infected cells concluded that many of these proteins might be of cellular origin and that the envelope of EAV contained only a single 21 kDa glycoprotein (Horzinek, 1981; Zeegers *et al.*, 1976). More recently, viral-specific proteins of 30 kDa, 42 kDa, and 60 kDa were identified in membranes from EAV-infected BHK21 cells, but not the 21 kDa glycoprotein (van Berlo *et al.*, 1986b). When present, the concentration of the 21 kDa protein was relatively low and it has not been proved that it is a viral protein. It seems more likely that a heterogeneously migrating set of proteins (28 kDa–42 kDa) represents the envelope glycoprotein(s) of EAV (den Boon *et al.*, 1991). Thus the structural proteins of EAV are very similar in number and size to those of LDV (Table I). Because of this resemblance it seems likely that the 28 kDa–40 kDa glycoproteins represent a single gene product that differs in molecular weight due to different degrees of glycosylation, as suggested for LDV (see Section II,C,1), but the possibility that they represent more than one gene product has not been ruled out. Recent sequence analyses (see below) indicate that the EAV genome encodes four potential glycoproteins of 25.6 kDa, 18.0 kDa, 17.2 kDa, and 28.7 kDa that seem to be translated from subgenomic mRNAs 2,3,4, and 5, respectively (den Boon *et al.*, 1991) (Fig. 11). These proteins possess one, six, three, and one potential glycosylation sites, respectively, but which of these proteins are envelope proteins and which nonstructural proteins has not been resolved (den Boon *et al.*, 1991). Although EAV has many features in structure and composition in common with LDV, it is serologically unrelated to LDV (van Berlo *et al.*, 1983), and on the basis of the ORFs of their genomes, there exists only very limited amino acid sequence homology between the proteins of LDV and EAV (see Secion II,C2 and below).

The genome organization of EAV, like that of LDV, is similar to that of coronaviruses (Spaan *et al.*, 1990; den Boon *et al.*, 1991). The formation of multiple molecular species of polyadenylated RNA in infected BHK21 cells was first indicated by results from [^{3}H]uridine-labeling in the presence of actinomycin D (Fig. 9) (van Berlo *et al.*, 1982). The labeled, virus-specific RNAs ranged in size from 0.8 to 12.7 kb, the molecular size of the genomic RNA, and the smaller two RNAs domi-

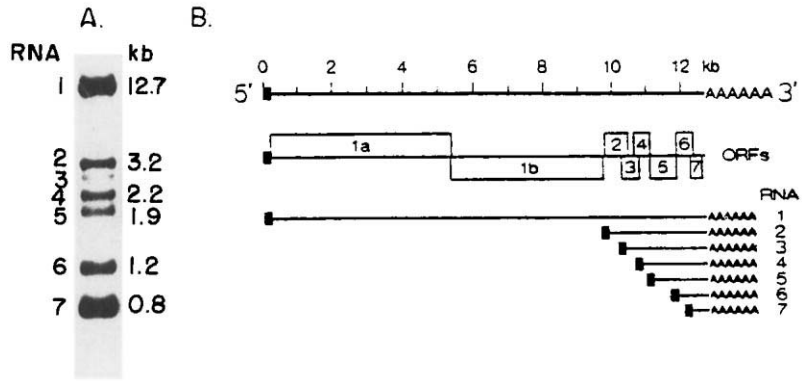

FIG. 11. Northern blot hybridization analyses of RNA extracted from infected BHK21 cells with a 3'-end probe (A) and genomic organization of EAV (B). [Data based on work of Spaan *et al.* (1990) and den Boon *et al.* (1991).] The boxes with numbers designate ORFs; the filled boxes represent the 5' leader sequence. For details, see text. (Reproduced with permission of the authors and publisher.)

nated at the time of maximum RNA synthesis. UV transcription mapping indicated that the UV target size of RNAs 2–6 were close to the physical size of the genomic RNA (van Berlo *et al.*, 1982). The results were consistent with a model in which RNAs 2–6 are derived from a larger percursor RNA molecule of the size of the genomic RNA. This model has been supported by results from subsequent studies, which demonstrated that the EAV RNAs represent a 3'-coterminal nested set of mRNAs (Fig. 11). *In vitro* translation experiments showed that the smallest RNA encodes VP-1 (van Berlo *et al.*, 1986b) A cDNA copy of this RNA hybridized in Northern blot analyses to all the polyadenylated RNAs isolated from infected cells (van Berlo *et al.*, 1986a) (Fig. 11A). Furthermore, sequence analyses and Northern blot hybridizations indicated that all subgenomic RNAs possess a common 5' 207-nt leader sequence that is encoded by the 5' terminus of the genomic RNA (Spaan *et al.*, 1990; de Vries *et al.*, 1990; den Boon *et al.*, 1991). A probe hybridizing to the 5' end of genomic RNA also hybridized to all the subgenomic mRNAs. The leader is linked to the body of each mRNA by a common 5'-UCAAC-3' sequence that is located at various distances upstream of the translation initiation codon of each mRNA (de Vries *et al.*, 1990; den Boon *et al.*, 1991). Some ORFs are preceded by two or three of these sequences. Only one seems to function as leader–body junction site, but which one is uncertain.

The genome of the Bucyrus strain of EAV has recently been completely sequenced (den Boon *et al.*, 1991). The length of the genomic RNA is 12.7 kb and the length of subgenomic mRNAs 2–7 are 3.2, 2.7, 2.2, 1.9, 1.2, and 0.8 kb, respectively (Fig. 11). Each subgenomic mRNA possesses a single ORF. Subgenomic mRNA 7 encodes VP-1 and mRNA 6 probably encodes the envelope protein VP-2, because it encodes a protein of the appropriate molecular weight, which is nonglycosylated and contains three potential membrane-spanning segments. ORFs 2, 3, 4, 5, 6, and 7 overlap with each other and each ORF is read in different frame than those on either side of it. The overlap regions range from 9 nt (ORFs 4 and 5, and 5 and 6) to 198 nt (ORFs 2 and 3). The gene products of the other subgenomic mRNAs 2–5 have not been identified (see above). As is the case for the coronaviruses and toroviruses, the subgenomic mRNAs of EAV are transcribed from only a small portion of the total viral genomic RNA (only about 3 kb of the total of 12.7 kb; see Fig. 11B). The 5′-terminal 10 kb of the genome encodes a single gene with two ORFs (1a and 1b), ORF 1b being in the −1 reading frame with respect to ORF 1a (Fig. 12). The overlapping region contains a "shift" ("slippery") heptanucleotide (5′-GUUAAAC-3′) and a potential

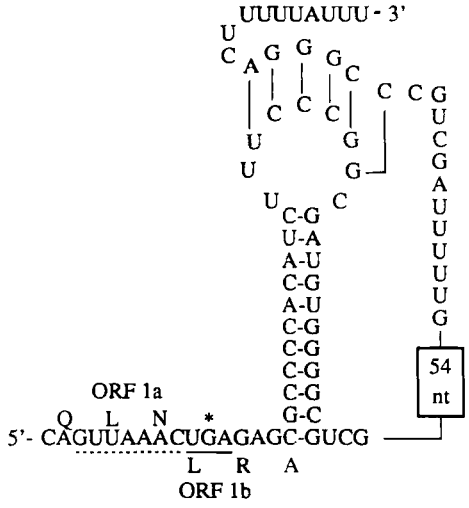

FIG. 12. Predicted secondary and tertiary RNA (pseudoknot) structure of the EAV ORF 1a/ORF 1b junction region. The slippery sequence G UUA AAC is indicated by a dashed line. The ORF 1a termination codon is underlined and the carboxy-terminal amino acids of ORF 1a and of the amino-terminal amino acids of ORF 1b are indicated. (Modified from den Boon *et al.*, 1991; presented with permission of the authors.)

downstream RNA pseudoknot structure (Fig. 12). These elements are similar to those found in coronavirus (Brierly *et al.*, 1989; Bredenbeck *et al.*, 1990) and torovirus (Snijder *et al.*, 1990) genomes, except that the slippery sequence of EAV diverges by 1 nt from the 5′-UUUAAAC-3′ sequence of the latter viruses. Evidence has been presented to indicate that ORF 1b of all these viruses is expressed by ribosomal frameshifting and that a slippery sequence and a pseudoknot are essential for efficient frameshifting (den Boon *et al.*, 1991). The frameshift-directing potential of the ORF 1a/1b overlap region was demonstrated by using a reporter gene (den Boon *et al.*, 1991) similar to that previously used for exploring the frameshift mechanisms for MHV-A59 (Bredenbeck *et al.*, 1990) and BEV (Snijder *et al.*, 1990).

As discussed already (Section II,C,2), ORF 1b contains an RNA replicase motif (Fig. 8) that greatly resembles that of LDV and to a lesser extent those of the coronaviruses and BEV. ORF 1b of EAV (1448 amino acids) is considerably smaller than ORF 1b of the coronaviruses and toroviruses. However, it contains three other motifs (motifs 2–4) (den Boon *et al.*, 1991) that correspond to similar motifs of the ORF 1b of the coronaviruses (Bredenbeck *et al.*, 1990) and BEV (Snijder *et al.*, 1990). These are a zinc finger motif (2), a helicase motif (3), and a conserved motif (4) near the C terminus of ORF 1b, which was initially found to be characteristic and unique for both coronavirus and torovirus genomes (Snijder *et al.*, 1990). Considerable greater amino acid divergence is apparent in the zinc finger and helicase motifs of the 1b proteins of EAV, coronaviruses, and toroviruses than in the replicase motif. EAV ORF 1a (1727 amino acids) is also much smaller than the ORF 1a of coronaviruses and toroviruses and no domains of significant homology are apparent (den Boon *et al.*, 1991). EAV ORF 1a contains three cysteine-rich motifs in the amino-terminal part of the protein, some very hydrophobic regions, and a serine proteinase consensus sequence (den Boon *et al.*, 1991). Similar putative proteases are found in the coronavirus and torovirus 1a proteins, but their potential functions in processing the primary ORF 1a and ORF 1b translation products into smaller active units are unexplored.

How the subgenomic mRNAs are produced remains to be elucidated. The observation that they seem to express similar UV target sizes could suggest that they may be formed by the splicing of a common precursor (Spaan *et al.*, 1990). However, RNA splicing reactions seem to be restricted in mammalian cells to the nucleus and there is no evidence to indicate a nuclear phase in the replication of EAV. Furthermore, the sequences at the junction sites of the EAV RNA leader and the body of the mRNAs are not compatible with sequences involved in pre-mRNA

splicing, splicing of tRNAs, and self-splicing RNAs with group II introns (Spaan *et al.*, 1990). However, the leader sequence flanking the 5′-UCAAC-3′ junction between the leader and the body of the mRNAs is very similar to the 5′ splice site of *Tetrahymena* pre-rRNA (Spaan *et al.*, 1990; de Vries *et al.*, 1990). Formation of subgenomic mRNAs via a splicing process would be a novel mechanism for RNA viruses and differ from the leader-primed mRNA synthesis postulated for coronaviruses (Spaan *et al.*, 1990), with which EAV shares its genome organization. Thus additional studies are necessary to clarify the mechanism of synthesis of the subgenomic mRNAs of EAV. Also of interest is the question of whether the subgenomic RNAs are formed in the course of minus-strand or plus-strand RNA synthesis or both, a question also not resolved for coronaviruses (Hoffmann *et al.*, 1990; Sethna *et al.*, 1989; Sawicki and Sawicki, 1990).

EAV is presently classified as the sole species of the genus *Arterivirus* of the family Togaviridae (Westaway *et al.*, 1985a). However, the foregoing information indicates that its genome organization and expression is more closely related to those of the coronaviruses and toroviruses than to those of other members of the Togaviridae.

IV. Simian Hemorrhagic Fever Virus

SHFV was first isolated from diseased macaque monkeys during devastating outbreaks of hemorrhagic fever that occurred almost simultaneously in 1964 in the Soviet Union and the United States (Tauraso *et al.*, 1968a,b). Information on the morphology and biochemical properties of SHFV reported before 1980 has previously been summarized by Brinton (1980) and Horzinek (1981). The considerable new findings concerning the pathogenesis and molecular properties of this virus, reported during the last 10 years, will be the main focus of this review.

A. Epizootics

The first recorded epizootics occurred in a macaque monkey colony of the Institute of Experimental Pathology and Therapy at Sukumi, U.S.S.R., and at the NIH quarantine colony at Bethesda, Maryland (Lapin *et al.*, 1967; Palmer *et al.*, 1968). The monkeys had been shipped from India to both institutions by the same supplier. In the outbreak at the NIH facilities, 223 macaque monkeys became ill and practically all died within a 2-month period (Palmer *et al.*, 1968). Similar epizootics

occurred subsequently in macaque monkey colonies at the National Center for Primate Biology at Davis, California in 1967, at a primate colony in Sussex, England between 1966 and 1969, at the NIH facilities in 1972 (London, 1977; Trousdale *et al.*, 1975), and most recently in 1989 at the Primate Research Center, Alamogorda, New Mexico, at Hazelton Research Products, Reston, Virginia, and at the shipping company in Miami, Florida (Jahrling *et al.*, 1990; R. O. Cannon, Center of Disease Control, personal communication). The recent outbreaks were among cynomolgus monkeys (*Macaca fascicularis*) shipped to the United States from two suppliers in the Philippines, but the infections spread to other macaque monkeys in these facilities. Several hundred macaque monkeys died during these outbreaks. In one outbreak at Hazelton Research Products, Ebola virus was also detected and isolated from some ill monkeys and it is uncertain to what extent the epizootics were caused by each of the viruses or a combination of the two (Jahrling *et al.*, 1990). In each of the outbreaks cited above, nearly all monkeys in the affected macaque populations either died or were euthanized.

B. Pathogenesis

The onset of the disease in macaques is rapid and consists of early fever, mild facial edema, anorexia, adipsia, dehydration, proteinuria, cyanosis, skin petechia, bloody diarrhea, nose bleeds, and occasional hemorrhages in the skin (Palmer *et al.*, 1968; London, 1977). Death generally occurs between 5 and 25 days after onset of the illness and mortality approaches 100%. Very few macaque monkeys seem to survive the infection (Tauraso *et al.*, 1968a). Once illness becomes apparent in the macaque colony the disease spreads rapidly through the colony. The mode of transmission has not been elucidated but probably occurs by contact and intranasally via aerosols (London, 1977). The virus is readily transmitted to other macaque monkeys by intramuscular or intracranial injections of blood from a diseased monkey (Palmer *et al.*, 1968).

Pathological lesions are widespread and consist of capillary–venous hemorrhages in the intestine, lung, nasal mucosa, dermis, spleen, perirenal and lumbar subperitoneum, adrenal glands, liver, and periocular connective tissues (Allen *et al.*, 1968; Abildgaard *et al.*, 1975; London, 1977). The lesions are often associated with evidence of vasodilation, stasis, and venous thrombosis. Thus, shock is suspected as the underlying causative factor (Allen *et al.*, 1968), which is perhaps related to the replication of SHFV in macrophages (see later).

During the first outbreak at the NIH facilities it became apparent

that the susceptibility of monkeys to the devastating disease might be limited to macaque monkeys (Palmer *et al.*, 1968). No clinical symptoms were apparent among 50 young patas monkeys (*Erythrocebus patas*), a small number of squirrel monkeys (*Saimiri sciureus*), and chimpanzees (*Pan satyrus*). In contrast, the disease rapidly spread from initially infected rhesus monkeys (*Macaca mulatta*) to cynomolgus and stump-tailed macaque (*Macaca arctoides*) monkeys. Thus, all species of macaque monkeys are highly susceptible and macaques seem to be the only genus of monkeys that develop this severe and usually fatal disease after infection with SHFV (London, 1977; Gravell *et al.*, 1986a).

Epizootics in colonies of captive macaque monkeys probably originate mainly or exclusively from accidental transmission of SHFV from monkeys of other genera that are persistently infected with SHFV without exhibiting clinical symptoms (London, 1977). A survey of patas monkeys caught in the wild and imported from Africa indicated that 49% of a total of 216 tested were persistently infected as demonstrated by isolation of the virus from these monkeys by inoculation of their blood into cultures of rhesus monkey macrophage cultures (see later) or into susceptible rhesus monkeys (Gravell *et al.*, 1980c). No clinical disease was observed in patas monkeys, in spite of continuous viremia. In the wild, African monkeys of three genera, namely patas monkeys, African green monkeys (*Ceropithecus aethiops*), and baboons (*Papio anuibus* and *Papio cyanocephalus*), seem to carry the virus in a persistent, asymptomatic state (London, 1977; Gravell *et al.*, 1986a). No information is presently available on the morbidity and mortality of infected monkeys of these genera or how frequently and widespread persistent infections are in their natural habitat. Infections of captive monkeys of these genera with SHFV result in persistent infection in the absence of any clinical symptoms or in transient mild disease, depending on the virulence of the inoculated strain (London, 1977; Gravell *et al.*, 1986a; see below). In the latter case, the clinical symptoms resemble those observed in infected macaque monkeys, except that they are milder, and only a few patas monkeys have died after experimental infection with one of the more virulent strains of SHFV (see below).

The 1972 NIH outbreak at the NIH facilities, in which 212 macaques were lost within 12 days, has been traced to persistently infected viremic patas monkeys (London, 1977). The accidental mechanical transmission of the virus from these monkeys to susceptible macaque monkeys was attributed to poor animal care techniques. Most likely it involved injection of both types of monkeys from a multidose vial with a common needle and syringe (London, 1977; Gravell *et al.*, 1986a). Another possible mode of transmission is via tatooing animals without

decontaminating the needle between each procedure (London, 1977). How the virus is transmitted between patas, African green, and baboon monkeys in endemic areas in Africa is uncertain. The virus is not transmitted transplacentally from mother to offspring (Gravell *et al.*, 1986b). No insect vector has been identified, but the virus most likely can infect through open wounds like most blood-borne viruses and can be transmitted by biting (Gravell *et al.*, 1986b).

The source of virus in most of the epizootics, however, has not been proved. Disease developed in macaque monkeys shortly after they were imported from Asia (London, 1977; Jahrling *et al.*, 1990), but when and how they became infected is unclear. No natural epizootics of SHFV in macaque monkeys have been reported. Obviously, natural transmission of SHFV from persistently infected African monkeys to macaque monkeys with residence in Asia is excluded because of the geographical separation of these genera of monkeys.

With respect to the most recent epizootics in the United States, in 1989, one group of 100 cynomolgus monkeys was shipped from the Philippines via Amsterdam, and contact with persistently infected African monkeys either in the Philippines or during transit cannot be ruled out. On the other hand, a SHFV-like virus has been isolated from wild mice at one of the monkey facilities in the Philippines from which the monkeys were shipped and this isolate caused characteristic hemorrhagic fever in injected macaque monkeys (P. B. Jahrling, personal communication). Thus, the existence of alternate hosts as a source of infectious virus needs to be reevaluated.

C. Propagation in Cell Culture and Identification of Isolates Varying in Virulence

Although hemorrhagic fever could be readily transmitted to the macaque monkeys by injection of serum from diseased animals, isolation of the causative virus was initially problematic. No infections resulting in disease could be established in various laboratory animals, such as mice, rats, hamsters, and guinea pigs, in embryonated chicken eggs, or in different types of cell cultures (Lapin *et al.*, 1967; Tauraso *et al.*, 1968a). Cytopathic effects were eventually observed in cultures of one line of monkey kidney cells, MA-104 (also referred to as USU-104; Gravell *et al.*, 1986a), which had been inoculated with tissue extracts from a diseased stump-tailed macaque monkey of the 1964 NIH epizootic (Tauraso *et al.*, 1968a). The MA-104 line was originally thought to be derived from rhesus monkeys, but recent immunological evidence indicates that it originated from African green monkeys (M. Gravell,

personal communication). Viral yields in the cultures of MA-104 cells seemed to increase with successive passages in the cells. This isolate, LVR42-0/M6921, represents the prototype strain of SHFV and is generally referred to as LVR (Table V). Several other strains were isolated in MA-104 cultures from a total of 11 different monkeys during the 1964 NIH epizootic (Tauraso *et al.*, 1968a). All strains produced similar cytopathic changes in MA-104 cultures (Tauraso *et al.*, 1968a). The findings led to the establishment of an end-point dilution assay (Tauraso *et al.*, 1968a) and a plaque assay (Gravell *et al.*, 1980b) in MA-104 cell cultures for the titration of SHFV, but most data available have been obtained by the end-point dilution assay. Viral yields in infected cultures were about 10^6–10^7 TC ID_{50}/ml of culture fluid. SHFV LVR has also been propagated in another line of African green monkey kidney cells, BSC-1 (Wood *et al.*, 1970).

TABLE V

COMPARISON OF CHARACTERISTICS OF LVR, P-180, P-248, AND P-741 ISOLATES OF SHFV[a]

Characteristic	Isolate			
	LVR	P-180	P-248	P-741
Origin	Diseased rhesus monkey (NIH, 1964)	Diseased patas monkey (NIH, 1972)	Asymptomatically infected patas monkey	Diseased rhesus monkey, experimentally infected
Type of infection in macaque monkeys	Acute, fatal hemorrhagic disease	Acute, fatal hemorrhagic disease	Acute, fatal hemorrhagic disease	Acute, fatal hemorrhagic disease
Type of infection in patas monkeys	Acute disease, usually nonfatal	Severe acute disease, sometimes fatal	Asymptomatic persistent infection	Asymptomatic persistent infection
Antigenic relationship	Related	Related	Most distantly related	Related
IgG antibody response in patas monkeys	High	High	Low	Low
Cytopathology				
MA-104 cells	Lytic	Nonlytic	Nonlytic	Nonlytic
Patas PMs[b]	Lytic	Lytic	Nonlytic	Nonlytic
Rhesus PMs	Lytic	Lytic	Lytic	Lytic

[a] Data are modified from Gravell *et al.* (1986a).

[b] PM, Peritoneal macrophage.

An isolate similar to the LVR strain has been obtained by inoculation of MA-104 cell cultures with tissues from a macaque monkey that contracted the disease during the 1964 Sukhumi outbreak (Tauraso *et al.*, 1968b). The various isolates available at the time cross-reacted in a complement fixation assay (Tauraso *et al.*, 1968b). In addition, a similar SHFV isolate was derived from a macaque monkey of the 1969 Sussex outbreak, but it became apparent that the efficiency of isolation of SHFV from different sources in MA-104 cell cultures varied greatly (Myers *et al.*, 1972). Moreover, SHFV isolated from asymptomatic, persistently infected patas monkeys, though highly virulent for macaque monkeys, was found not to induce cytopathic effects in MA-104 cultures (London, 1977; Gravell *et al.*, 1980a,b). One isolate from asymptomatic patas monkeys (P-248; see Table V), however, established a persistent infection in MA-104 cell cultures without causing any obvious cytopathic changes (Gravell *et al.*, 1980b). Because the isolate seemed to infect only a low percentage of the cells in these cultures (see Fig. 13A), the destruction of infected cells could have been missed in the presence of continued cell proliferation. Further work is required to elucidate the nature of the persistent infection of MA-104 cell cultures with P-248 SHFV. No interferon production or defective interfering particles were detected in persistently infected MA-104 cultures (Gravell *et al.*, 1980b).

In contrast, it was observed that the P-248 isolate caused a lytic infection in primary cultures of rhesus monkey peritoneal macrophages (PMs) (Gravell *et al.*, 1980a). This finding led to a more detailed comparison of the pathogenic properties of SHFV isolates that had been derived from various sources (Gravell *et al.*, 1986a). Besides the prototype strain LVR and the P-248 isolate, two other isolates were analyzed (Table V): P-180 isolated from the spleen of a patas monkey that died during the 1972 NIH epizootic and P-741 isolated from a rhesus monkey that died after experimental infection with serum from an asympotomatic, persistently infected patas monkey (Gravell *et al.*, 1986a). The species of origin of LVR and P-180 could not be specified because they were isolated from macaque monkeys that had become infected from unknown sources. The summary in Table V shows that the four isolates differed greatly in their virulence for rhesus and patas monkeys as well as for different types of cells in culture. All four isolates had essentially the same virulence for rhesus monkeys, causing acute fatal hemorrhagic disease, but two of them (P-248 and P-741), originally derived from persistently infected patas monkeys, caused little or no disease in patas monkeys, whereas the other two (LVR and P-180) caused more severe, though generally not fatal, infections in patas

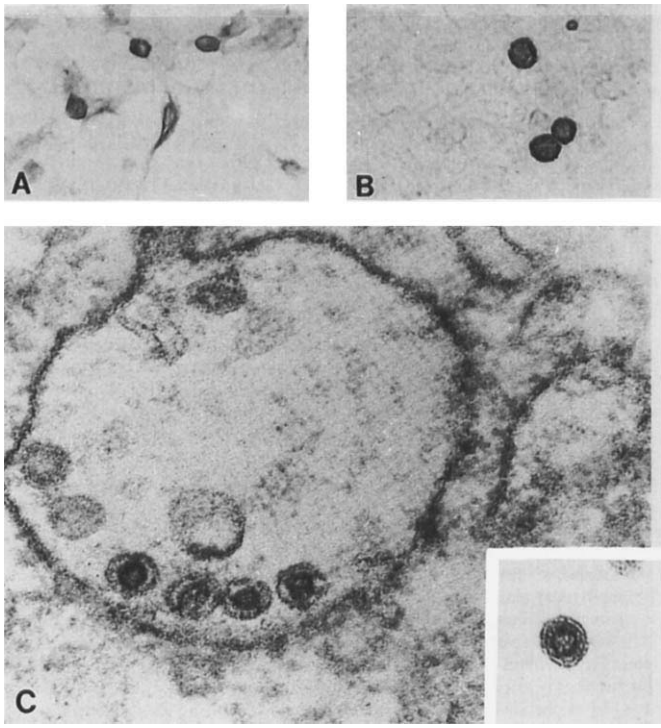

FIG. 13. Immunocytochemical staining of MA-104 cultures cytolytically infected with LVR SHFV for 20 hr (a) or persistently infected with P-248 SHFV (b) and electron micrograph of a section of MA-104 cells persistently infected with P-248 SHFV (c). The LVR virion is shown for comparison in the insert (c) (Data from Gravell *et al.* 1980b; reproduced with permission of the authors and publisher.)

monkeys. All of the isolates caused lytic infections of rhesus monkey PM but P-248 and P-741 had no effect on patas monkey PM or MA-104 cells. On the other hand, P-180 also caused a lytic infection in patas monkey PMs, but not in MA-104 cells, whereas LVR caused a lytic infection in all three cell types. The virus isolates also differed in their immunogenicity in patas monkeys and to some extent in serological specificity (see later).

The distinct virulence properties of these isolates seemed to be quite stable (Gravell *et al.*, 1986a). For example, SHFV P-248 retained its virulence for macaque monkeys during long-term cultivation of persistently infected MA-104 cells without gaining the ability to establish a lytic infection in patas monkey PMs or MA-104 cells (Gravell *et al.*,

1986a) (Table V.) These results indicate that the variants preexisted in populations of naturally infected monkeys and did not arise as a result of selection of mutants generated during the epizootic infection. However, further studies on this point seem desirable because no information is presently available on how different variants arise in nature and what selective pressures might be operating in their selection. Nevertheless, the results suggest that variants with low virulence and immunogenicity (see later) for patas monkeys have become selected in persistently infected monkeys of this species and perhaps other African species.

It has been suggested that macrophages are the primary target cell for SHFV in macaque monkeys (Gravell *et al.*, 1986a,b) and that there might exist a causal relationship between the cytocidal infection of these cells by SHFV and the clinical symptoms of hemorrhagic fever. Macrophages may also be the main target cell in patas monkeys for SHFV isolates that cause acute disease in this species, such as P-180 and LVR, because these isolates cause lytic infection of patas monkey PMs (See Table V). The target cells for SHFV in persistently infected patas monkeys are less clear, because the variants isolated (P-248 and P-741) from such monkeys fail to infect primary cultures of patas monkey PMs (see Table V and Fig. 13). Continuous SHFV replication, however, must occur in persistently infected patas monkeys because persistent viremia of 10^3–10^5 TC ID_{50}/ml serum has been observed in these animals (Gravell *et al.*, 1986a). Perhaps the variants in persistently infected patas monkeys replicate cytocidally in a subpopulation of macrophages that is continuously regenerated in the animal, just as is observed in persistent LDV infections of mice (see Section II,A,1), or the patas monkey macrophages are susceptible to infection by these variants only in specific stages of differentiation (Gravell *et al.*, 1986b). In either case, the SHFV-permissive population of macrophages might not be represented in cultures of peritoneal exudate cells of these monkeys, or the permissive cells lose their surface component that acts as SHFV receptor during *in vitro* culture.

Replication of SHFV in cell cultures is rapid; release of progeny virus begins about 4 hr postinfection (at 37°C) and virus production is practically complete by 8–10 hr postinfection. This virus growth curve has been demonstrated for the replication of LVR SHFV in MA-104 cell cultures (London, 1977) and for P-248 SHFV in rhesus monkey PM cultures (Gravell *et al*, 1986a) (Fig. 14). Figure 14 also illustrates that the P-248 isolate failed to replicate significantly in primary cultures of patas monkey PMs. Cytopathic effects become apparent in productively infected cultures 12–15 hr postinfection.

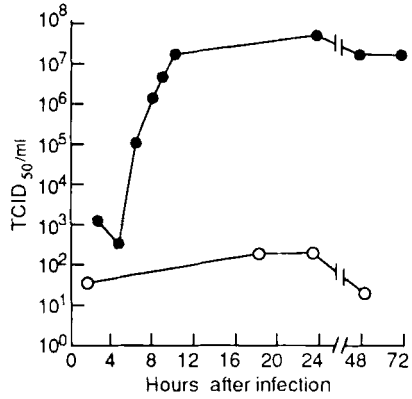

FIG. 14. Replication of the P-248 variant of SHFV in rhesus (●) or patas monkey PMs (○). PM cultures in 25-cm^2 vessels were infected with about 1 $TCID_{50}$/cell of P-248 virus. Cultures were incubated at 37°C and samples of medium were removed periodically and were titrated by an end-point dilution assay in primary cultures of rhesus monkey. PMs (Data from Gravell *et al.* 1986a; reproduced with permission of the authors and publisher.)

D. Host Immune Responses and Virus Clearance

A complement fixation assay using concentrated LVR SHFV propagated in MA-104 cell cultures indicated that some macaque monkeys that survived an experimental infection with the virus developed antiviral antibodies (Tauraso *et al.*, 1968a,b). A single macaque monkey that survived the 1964 NIH epizootic developed chronic illness, which was associated with both continuous low-level viremia and the presence of complement-fixing anti-viral antibodies. In general, however, the rapidity of the acute infection and death of macaque monkeys preclude an effective host immune response.

The response of patas monkeys is different from that of macaque monkeys and has not been entirely resolved. Anti-SHFV serum antibody levels as measured by ELISA using MA-104 cell culture-propagated LVR as antigen varied greatly in persistently infected patas monkeys (Gravell *et al.*, 1986a). ELISA antibody titers ranged from $\leq$ 10 to 6250 in individual patas monkeys that had become naturally infected. Results from experimental infections of patas monkeys indicated that these variable antibody responses might be explained by dual infections of some of the monkeys with SHFV variants that differed in virulence and immunogenicity (Gravell *et al.*, 1986a). For example, infection of patas monkeys with variants P-248 and P-741,

which had been originally isolated from persistently infected patas monkeys, resulted in asymptomatic persistent infections with low-level but persistent viremia of between 10^3 and 10^5 $TCID_{50}$/ml of serum and minimal or no significant anti-SHFV antibody response. No neutralizing antibodies were detected in these monkeys. In contrast, infection of patas monkeys with the more virulent LVR isolate induced acute disease, which was associated with high levels of viremia of 10^6–10^7 $TCID_{50}$/ml of serum 3–6 days postinfection and the formation of serum anti-LVR antibodies beginning about 7 days postinfection. The formation of anti-LVR antibodies correlated with the complete clearance of the virus from the circulation by 21 days postinfection (Gravell *et al.*, 1986a). Superinfection of one virus-free monkey with the P-248 variant at 48 days postinfection resulted in the establishment of persistent infection, in spite of the presence of high serum levels of anti-LVR antibodies. This outcome seems to be explained by the finding that the LVR and P-284 isolates cross-reacted little, if at all, in ELISAs using each strain purified from cell cultures and serum from monkeys infected with these strains (Gravell *et al.*, 1986a) (Table V). The implication of these results was that antibodies that neutralize the LVR isolate do not significantly neutralize the P-248 isolate. The more virulent LVR and P-180 variants, on the other hand, are closely related antigenically, whereas the P-741 isolate is related more remotely (Table V).

In summary, a spectrum of SHFV variants that vary greatly in virulence and immunogenicity for patas monkeys seem to exist in nature. The more virulent strains replicate efficiently in macrophages of these monkeys, causing acute infection, which is associated with high levels of viremia but which is eventually cleared by an effective antiviral immune response, probably via antibody-dependent cellular cytotoxicity (Gravell *et al.*, 1986a). In contrast, the replication of the less virulent SHFV variants seems more restricted in patas monkeys to an as-yet unidentified cell type and the variants seem to be less immunogenic. Infection with these variants leads to an asymptomatic persistent infection associated with relatively low levels of viremia and a lack of formation of neutralizing antibodies. Differences of variants in virulence, cell tropism, and immunogenicity seem to be associated with differences in antigenic specificity, but it is uncertain to what extent the differences in these properties are related to each other or are responsible for the ability of the variants to establish acute or probably lifelong persistent infections in patas monkeys. In spite of these differences in patas monkey infections, all variants cause acute, generally fatal hemorrhagic disease in macaque monkeys associated with the variants' replication in macrophages.

Less readily explained on the basis of the scenario outlined above are the events that occurred in patas monkeys that were superinfected with the more virulent LVR or P-180 variants after being persistently infected with the P-248 isolate for 147 days. The persistent infection terminated with complete clearance of both the persistent virus and the superinfecting virus in the absence of any formation of complement-dependent or -independent anti-P-248 antibodies (Gravell *et al.*, 1986b). This finding has been observed in 20 patas monkeys. Moreover, reinfection of two of the monkeys after elimination of all infectious SHFV with the P-248 variant resulted in only a transient, short-term viremia followed by complete clearance of the virus (Gravell *et al.*, 1986b). These results indicate that the clearance of the P-248 variant from the persistently infected animals was mediated by immune mechanisms, even though antibodies to LVR and P-180 do not neutralize the P-248 variant. Perhaps the P-248 variant was cleared by CTLs that might be generated in patas monkeys only when dually infected simultaneously with this variant and a more immunogenic and more virulent variant.

Because persistently infected patas monkeys represent a potential source of virus for initiating devastating epizootics in macaque colonies, it seems important to identify and eliminate such monkeys (Gravell *et al.*, 1986b). However, serological tests do not seem reliable for their detection because of the low levels of anti-SHFV antibodies found in many persistently infected patas monkeys. Persistently infected monkeys can be identified by the presence of virus that replicates in cultures of rhesus monkey macrophages (Table V), but persistently infected monkeys have been missed by this method (Gravell *et al.*, 1986b). Macaque inoculation still appears to be the most sensitive method for detecting persistently infected monkeys, but this method is very expensive (Gravell *et al.*, 1986b). Perhaps less expensive molecular techniques can be developed for SHFV detection once the sequence and organization of its genome have been determined (see Section III,A).

E. Molecular Properties

Electron microscopic studies of the LVR isolate of SHFV propagated in MA-104 cell cultures indicated that its structure is similar to those of LDV and EAV, although the diameters of the virion and its core seem to be slightly smaller than those of the other two viruses, namely 40–45 and 22–25 nm, respectively (Wood *et al.*, 1970) or 45–50 and 25nm, respectively (Trousdale *et al.*, 1975) (Table I). Similar particles were observed in MA-104 cells infected with the P-248 variant, but the

diameter of the virions was not estimated (Gravell *et al.*, 1980b) (Fig. 13c). Togavirus-like particles with a diameter of about 60 nm and a core with a diameter of about 30 nm have been detected in the placenta of a baboon and the urinary bladder of a capuchin monkey, but the nature and origin of these particles were not determined (Smith *et al.*, 1978).

LVR-SHFV exhibits a buoyant density in sucrose density gradients of about 1.15 g/cm^3 (Leon-Monzon *et al.*, 1991) and 1.18 g/cm^3 in sodium-tartrate gradients (Trousdale *et al.*, 1975). Its sedimentation coefficient is 214 S and that of the nucleocapsid released by treatment with nonionic detergents is 174 S (Sagripanti, 1984a). The virions contain 49 S (5.5 × 10^{6}Da) single-stranded RNA (Sagripanti, 1984b), which is polyadenylated (Sagripanti, 1985). The mean length of the poly-A segment was found to be 76 ± 2 nucleotides. However, on the average, only 36% of the RNA isolated from virions labeled with [^{32}]Pi, [^{3}H]uridine, or [^{3}H]adenine bound to oligo(dT)-cellulose (Sagripanti, 1985). On the basis of the reported experiments it cannot be decided whether the genomic RNA of a high proportion of virons, when produced in cells, lacks poly(A) sequences of sufficient length to bind to oligo(dT), whether the poly(A) segment became lost during the isolation of the RNA, or whether the lack of binding of the uniformly labeled viral RNA was due to partial degradation of the RNA. The viral RNA carries a type I cap in the form of m7G(5')ppp(5')Am (Sagripanti *et al.*, 1986). SHFV RNA synthesis is not inhibited by actinomycin D (Sagripanti, 1984b).

The structural proteins of SHFV seem similar to those of LDV and EAV, but have not been entirely resolved. SDS–PAGE profiles of the ^{14}C-labeled amino acids of proteins of the LVR isolate propagated in MA-104 cells (Trousdale *et al.*,., 1975) strongly resembled those of the stuctural proteins of LDV and EAV. Three major proteins were observed, SP4, SP3, and SP1, which seemed to correspond to VP-1, VP-2, and VP-3 of LDV, respectively (see Section II,C,1). However, because no molecular weights for the SHVF proteins were recorded and the locations of the proteins within the virion were not determined, no direct comparisons were possible. Nevertheless, just as observed for LDV, SP1 (VP-3) was the main glycosylated protein of SHFV as determined by labeling with [^{3}H]glucosamine (Trousdale *et al.*, 1975). In one recent study, the three structural proteins of SHFV were identified as a 12-kDa nucleocapsid protein (N or VP-1), a 16- to 18-kDa nonglycosylated envelope protein (M or VP-2), and an envelope glycoprotein (E or VP-3) of about 50 kDa (Leon-Monzon *et al.*, 1991). Another study yielded values of 15 and 19 kDa for VP-1 and VP-2, respectively, and of 39 and 45 kDa for envelope glycoproteins (Godeny and Brinton, 1991).

No antigenic relationship was detected between SHFV, LDV, flaviviruses, pestiviruses, and alpha and rubi togaviruses (Leon-Monzon *et al.*, 1991).

SHF has been placed in the Flaviviridae family (Westaway *et al.*, 1985b), but recent preliminary results suggest that its genome organization resembles that of LDV and EAV; the VP-1 gene seems to be located at the 3' end of the genome and VP-1 surprisingly exhibits considerable homology with VP-1 of LDV (Godeny and Briton, 1991). SHFV also resembles to a greater extent LDV and EAV than the flaviviruses in its density, the nature of its structural proteins, and the size and polyadenylation of its genome (Table I). Furthermore, its replication cycle is similar to that of LDV and EAV and thus much shorter than those of the flaviviruses. The host range of SHFV is also narrower than that of flaviviruses and no alternate insect host has been identified.

V. Common Properties of Lactate Dehydrogenase-Elevating Virus, Equine Arteritis Virus, and Simian Hemorrhagic FeverVirus

The three viruses have many properties in common both at the molecular level and in the infection of their natural hosts, but there exist also some fundamental differences. First, in ultrastructure, the sedimentation coefficient and density of their virions, the sedimentation coefficient of their nucleocapsids, and the size (12–13Rb) and polyadenylation of their genomic RNA, the three viruses seem indistinguishable (Table I). The same is the case for their structural proteins, except for some apparent differences in their envelope glycoproteins. Recent evidence indicates that EAV and LDV also possess a similar genome organization and replicate via a 3' nested set of six or seven subgenomic mRNAs. Preliminary results suggest that the genome organization of SHFV resembles that of EAV and LDV.

Second, the primary target cell of all three viruses in their respective hosts are macrophages. This fact has been most clearly established for LDV. In the case of EAV and SHFV it has not been resolved whether other types of cells in their respective hosts may also become productively infected. Especially in the case of SHFV the cells supporting SHFV replication in persistently infected patas monkeys have not been identified. The replication of LDV and SHFV *in vitro* is highly restricted to primary cultures of macrophages, at least that of many

variants of SHFV. The exception is EAV which is more promiscuous, replicating in many different cell lines.

Third, the gowth cycle of all three viruses is very similar and very rapid. Viral RNA and protein synthesis begin 3–4 hr postinfection and virus replication is complete by 10–12 hr postinfection. Productive infection by all three viruses is also rapidly cytocidal.

Fourth, all three viruses seem to be able to establish asymptomatic persistent infections in their natural hosts, but critical differences seem to exist in the mechanisms involved. In the case of LDV, infection of a mouse results in lifelong persistence of the virus because LDV replication in macrophages is unaffected by the host immune response and seems to be regulated only by the availability of permissive macrophages. In contrast, host immune responses effectively clear infectious virus from the circulation in SHFV-infected patas monkeys. Persistent infections are only established in these monkeys by SHFV variants that fail to induce an effective anti-viral immune response. The anti-EAV immune response also suppresses the replication of EAV in horses, but how and to what extent the virus establishes long-lasting, low-level persistent infections still need to be elucidated.

The exact molecular relationships between the three viruses and their ultimate classification await completion of the sequence analyses of the LDV and SHFV genomes and elucidation of their genomic organization. Because its genome organization resembles that of coronaviruses and toroviruses, EAV (and in extension LDV and SHFV) has been suggested to be classified, along with coronaviruses and toroviruses, in a new proposed superfamily (Spaan *et al.*, 1990; de Vries *et al.*, 1990). However, besides genome organization, EAV and LDV have little in common with coronaviruses and toroviruses. Morphologically EAV and LDV resemble togaviruses and flaviviruses rather than coronaviruses and toroviruses (Spaan *et al.*, 1988). They are much smaller than coronaviruses and toroviruses, have a smooth surface rather than large envelope peplomers, and they possess a nucleocapsid with cubical rather than helical symmetry. Furthermore, their genome size is less than about one-half of those of the coronaviruses and toroviruses. On the basis of these differences it might be appropriate to place EAV and LDV in a new, separate family, perhaps designated *Arteriviridae* or *Multiviridae*, in recognition of their mode of replication via a set of multiple mRNAs. This group of viruses may be much larger than presently thought. Other viruses of this group may have escaped detection because they generally do not cause clinical disease in their natural host as is characteristic for LDV, EAV, and SHFV. A potential

additional member of this group may be a newly discovered virus, referred to as Lelystad virus (Wensvoort *et al.*, 1991), which causes a mild respiratory disease of pigs and late abortions in sows. The disease was first referred to as mystery swine disease, but has been renamed porcine reproductive and respiratory syndrome (PRRS; Ohlinger *et al.*, 1991). The Lelystad virus resembles LDV, EAV and SHFV morphologically, in its structural proteins and its preferences for macrophages both *in vivo* and *in vitro* (Ohlinger *et al.*, 1991; Pol *et al.*, 1991).

VI. The Question of Emerging Viruses

After the appearance of AIDS the question of emerging viruses, i.e., viruses that suddenly cause devastating disease as a result of selection of mutants with increased virulence or altered host range, has been raised (Culliton, 1990). LDV, EAV, and SFHV are of considerable interest in relation to this question. All three viruses, though highly cytocidal viruses, can establish long-lasting asymptomatic infections in their natural hosts and exhibit great genetic variability affecting virulence and/or host cell range. LDV does not cause any clinical disease in mice because its replication is restricted to a subpopulation of apparently nonessential macrophages. Because of its cytopathogenic nature and its ability to escape all host immune defense mechanisms, any mutant with increased host cell range might cause severe disease in mice. In view of the genetic variability of LDV, such variants have perhaps arisen, but have not become established because of the inefficient transmission of LDV between mice and because any mouse in which such a variant arose many have been rapidly killed. Attempts to generate host-range variants of LDV by propagation in mixed cultures of mouse macrophages with rat macrophages or mouse macrophage cell lines have been unsuccessful so far (Oneyakaba *et al.*, 1989b, and P.G.W. Plagemann, unpublished observations 1991). The potential appearance of virulent variants during persistent infection of mice does not seem to have been investigated.

Although this has not been proved, the appearance of epizootics of equine arteritis in horses is probably related to the appearance of more virulent variants of EAV, and the existence of multiple variants of SHFV that differ in virulence for their natural host has been clearly established. SHFV is a prime example of a devastating disease that is triggered in all species of one genus of monkeys by a virus that causes only asymptomatic persistent infections in other genera of monkeys. In nature, the Asian macaque monkeys are protected from infection from

SHFV-infected African monkeys only by geographical barriers. The epidemiology of SHFV infections is further complicated by the recent tentative isolation of a SHFV-like virus from rodents in the Phillippines (P.B.P Jahrling, personal communication). In view of the reported considerable sequence homology in the VP-1 proteins of LDV and SHFV (Godeny and Brinton, 1991), such a virus would also be of great interest in regard to the evolutionary relationship between these viruses. The Lelystad virus could represent a newly emerged virus of the LDV-EAV-SHFV group since PRRS has only recently appeared, first in the U.S.A. in 1987 and then in Germany in 1990 (Ohlinger *et al.*, 1991). The origin of the virus and its potential evolutionary relationship to LDV or EAV, however, are presently unknown.

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