Virology 290, 11–20 (2001)
doi:10.1006/viro.2001.1139, available online at http://www.idealibrary.com on IDEAL®

Complexity of the Single Linear Neutralization Epitope of the Mouse Arterivirus Lactate Dehydrogenase-Elevating Virus

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Received March 22, 2001; returned to author for revision June 5, 2001; accepted August 1, 2001

Results from indirect ELISAs using synthetic peptides of various length that represent segments of the ectodomain of the envelope glycoprotein, VP-3P, of lactate dehydrogenase-elevating virus (LDV) showed that the primary neutralization epitope of LDV is located in a short linear hydrophilic segment in the center of the ectodomain. The epitope becomes slightly altered by amino acid substitutions in the ectodomain and inactivation of virions by various treatments. Neutralizing anti-VP-3P antibodies (Abs) to the epitope interact with the synthetic peptides only if they possess a certain conformation. When the peptides were immobilized on ELISA plates, neutralizing mAbs elicited to inactivated LDV and neutralizing Abs from infected mice bound best to the peptides that consisted of the full-length, 30-amino-acid-long ectodomain. The Abs bound poorly, if at all, to most of the shorter peptides when immobilized, whether truncated at the N- or C-end, but when in solution the same peptides strongly inhibited the binding of the Abs to immobilized full-length peptides. Thus, a conformation of the epitope required for Ab binding and (or) its steric accessibility were lost upon immobilization of the shorter peptides on ELISA plates. Abs raised in mice to peptide–bovine serum albumin conjugates reacted only with immobilized peptides in the indirect ELISA and failed to neutralize LDV. The neutralization epitope of the common LDV quasispecies, LDV-P and LDV-vx, is flanked by N-glycans that block the immunogenicity of the epitope and the neutralization of these LDVs. Abs to a second weakly immunogenic and probably discontinuous epitope appear in LDV infected mice about 1 month postinfection.

INTRODUCTION

The mechanism of neutralization of viruses by antibodies (Abs) is generally incompletely understood as are the structures of the viral epitopes recognized by the neutralizing Abs (Flint *et al.,* 2000; van Regenmortel, 1998; Whitton and Oldstone, 1996; Parren and Burton, 2001). The mouse arterivirus lactate dehydrogenase-elevating virus (LDV) provides a unique model for addressing these questions since (1) the virus seems to possess only a single neutralization epitope that is located on the short (about 30 amino acids long) ectodomain of the primary envelope glycoprotein, VP-3P (Li *et al.,* 1998); (2) a number of LDV variants with amino acid differences in their VP-3P ectodomains have been isolated (Chen *et al.,* 2000; Plagemann *et al.,* 1999); and (3) batteries of neutralizing mAbs that react with VP-3P have been generated in two laboratories (Coutelier *et al.,* 1986; Coutelier and van Snick, 1988; Harty and Plagemann, 1988). Furthermore, linear synthetic peptides consisting of the 30-amino-acid-long VP-3P ectodomains of some LDV quasispecies have been shown to strongly bind in an indirect ELISA all neutralizing monoclonal Abs (mAbs) that have been examined as well as antibodies from acutely and persistently infected mice (Li *et al.,* 1998; Plagemann *et al.,* 1999).

Two classes of LDV quasispecies have been identified, the common LDV-P and LDV-vx and the neuropathogenic laboratory mutants, LDV-C and LDV-v (Chen and Plagemann, 1997; Chen *et al.,* 1997, 1998). These four LDVs have been cloned by repeated end-point dilution in mice from LDVs (LDV-PLA, LDV-C-BR, and LDV-VIR) originally isolated from mice carrying different transplantable tumors and found to be mixtures of two or three of these LDV quasispecies. The VP-3P ectodomains of LDV-P and LDV-vx contain three large N-glycans (Faaberg and Plagemann, 1995; Faaberg *et al.,* 1995; see P3 and P6 in Table 1) that impede the immunogenicity of the neutralization epitope contained in the ectodomain and render these LDVs highly resistant to *in vivo* Ab neutralization (Chen *et al.,* 1999, 2000; Plagemann *et al.,* 1999). In contrast, the VP-3P ectodomains of LDV-C and LDV-v lack the two N-terminal N-glycans (see P5 in Table 1). This allows these LDVs to cytocidally infect the anterior horn neurons of C58 and AKR mice but incidentally renders the neutralization epitope on the VP-3P ectodomain highly immunogenic and the virions susceptible to *in vivo* Ab neutralization (Chen *et al.,* 1998, 1999, 2000). Neuropathogenic LDV-v has been found to be a genetic recombinant of the nonneuropathogenic LDV-vx that has specifically acquired the VP-3P ectodomain of LDV-C and thereby the phenotypic properties of this LDV (Li *et al.,* 1999; Plagemann *et al.,* 2001).

In order to further assess the structure and specificity

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0042-6822/01 $35.00

TABLE 1

Synthetic Peptides to LDV VP-3P

Synthetic peptide	LDV specific	Amino acid sequence[a]
		25 30 35 40 45 50 55 60
P1	P	C L S T E N A C A A G **N** S
P3	P	A C A A G **N** S S T K N L I Y **N** L T L C E L **N** V T G F Q Q H F
P4	None	A V A A D **N** S S T K N L T S **N** L T L C E L **N** V T G F Q Q H F
P5	C/v	A C V A G D S S T K N L I Y T S T L C E L **N** V T G F Q Q H F
P6	vx/a[b]	A C G A S **N** S S T K N L I K **N** L T L C E L **N** V T G F Q Q H F
P7	P	N A C A A G **N** S S T K N L I Y **N** L T L C E L
P8	P	A C A A G **N** S S T K N L I Y **N** L
P9	C/v	A G D S S T K N L I Y T S T L C E L N V
P10	P	**N** S S T K N L I Y **N** L T L C E L **N** V T G F
P11	C/v	S S T K N L I Y T S T L C E L **N** V T G F
P12	P	S S T K N L I Y **N** L T L C E L **N** V T G F Q Q
P13	P	S S T K N L I Y

[a] Amino acids 1 to about 27 of the ORF5 protein represent the signal peptide, and the first transmembrane segment begins at about amino acid 61 (Faaberg and Plagemann, 1995). Potential N-glycosylation sites are in boldface lettering and amino acids that diverge from the LDV-P sequence are underlined. P4 possesses amino acid differences not found in any LDV VP-3P sequence.

[b] LDV-a is another nonneuropathogenic LDV variant (Chen *et al.,* 1998).

of the LDV neutralization epitope, and the reasons for the differences in immunogenicity of the epitope and in sensitivity to Ab neutralization between the two classes of LDV, I have now expanded our repertoire of VP-3P ectodomain-specific peptides (Table 1) and have examined by indirect ELISA the binding of the neutralizing mAbs and Abs from mice infected with various cloned LDV quasispecies to these peptides. I have also examined to what extent the VP-3P ectodomain-specific peptides inhibit in solution the binding of the anti-LDV Abs to selected immobilized peptides. In addition, I have generated antibodies to peptides containing the neutralization epitope and examined their ability to neutralize LDV.

RESULTS

Epitope specificity of neutralizing anti-LDV mAbs

We have demonstrated previously that the neutralizing mAbs raised to a mixture of formalin-inactivated LDV-P and LDV-vx (LDV-PLA), 159-7, 12, 18, and 19 (see Table 2) reacted strongly and about equally in an indirect ELISA with synthetic peptides consisting of the 30-amino-acid-long ectodomains of LDV-P, LDV-C/v, and LDV-vx/a, P3, P5, and P6, respectively (see Table 1), whereas the nonneutralizing anti-VP-3P mAbs from this battery did not bind to these peptides (Li *et al.,* 1998). The neutralizing mAbs reacted only weakly with P4 containing 4-amino-acid differences not found in any LDV or the N-terminal short peptide P1 (see Table 1). Similarly four other neutralizing anti-VP-3P mAbs generated in another laboratory (see Table 2) reacted with P3, but poorly with P4 (Li *et al.,* 1998).

I have now compared the reaction of the eight neutralizing mAbs (Table 2) with the full-length peptides and various additional peptides having truncated N- or C-termini (Table 1). The results were very similar for the four mAbs in each set of neutralizing mAbs, whether the

TABLE 2

Neutralizing Anti-LDV VP-3P mAbs

mAb[a]	Isotype	Origin
159-7	IgG2a	BALB/C mouse immunized with formalin-inactivated LDV-PLA[c] (Harty and Plagemann, 1988)
159-12	IgG2b	
159-18	IgG2a	
159-19	IgG1	
B6503 E7[b]	IgG2a	129/Sv mouse immunized with UV-light-treated LDV-RIL[c] (Coutelier *et al.,* 1986; Coutelier and van Snick, 1988; Coutelier personal communication)
B6501 A4	IgG2a	
B6505 H9	IgG2a	
C3904 H12	IgG3	2-month LDV-RIL[c]-infected BALB/c mouse (Coutelier *et al.,* 1986; Coutelier and van Snick, 1988; Coutelier personal communication)

[a] Immunoblotting has shown that all mAbs react specifically with VP-3P (Coutelier *et al.,* 1986; Harty and Plagemann, 1988).

[b] For simplicity, these mAbs are referred to in the text as E7, A4, H9, and H12.

[c] LDV-PLA and LDV-RIL were found to be mixtures of LDV-P and LDV-vx (Chen and Plagemann, 1997).

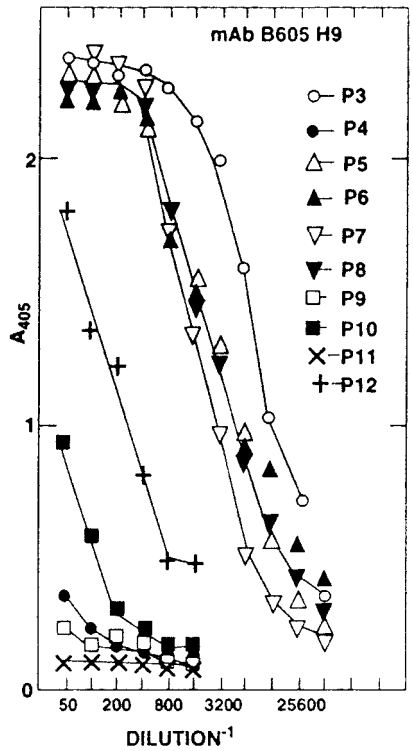

FIG. 1. Binding of neutralizing mAb B6505 H9 to peptides P3 to P12 as measured by indirect ELISA. All data were obtained in a single ELISA, which was conducted as described under Materials and Methods using the indicated peptides.

isotype of the mAbs was IgG2a, IgG2b, IgG3, or IgG1, but the reaction patterns were not identical. A typical binding pattern is presented for one mAb in Fig. 1 and for easier comparison the consensus results for each set of mAbs are summarized in Table 3. The results confirm that the mAbs reacted well with P3, P5, and P6, which encompass the amino acids present in the VP-3P ectodomains of all currently known LDV quasispecies, including those of four LDVs isolated from wild house mice (Li *et al.,* 2000). As found previously, all the neutralizing mAbs reacted very little, if at all, with P4 with four amino acid differences not found in any LDV. Significant binding was also observed to P7 and P8 in which the C-terminal end of the ectodomain was truncated (Fig. 1 and Table 3), but the mAbs bound poorly, if at all, to the peptides in which the N-terminal end was further truncated (P9–12).

The reactions of the sets of neutralizing mAbs with the various peptides differed slightly. One difference was that mAbs A4, E7, H9, and H12 reacted slightly better with P3 than with P5 and that the mAbs A4, E7, and H9 reacted better with P8 than the other mAbs (Table 3). In addition, mAb H12, but not mAbs A4, E7, and H9, reacted about equally with P3 and P6, while reacting less well with P7 and P8 than the latter.

Time courses of generation of epitope-specific Abs in infected mice

For comparing the peptide binding specificity of the Abs generated in infected mice, plasma must be obtained from these mice at a time when sufficient Abs have been generated to allow such comparison. Earlier studies had shown that high levels of Abs that bind to peptides P3 and P5 are generated very rapidly in LDV-C and LDV-v infected mice, reaching maximum levels at 3

TABLE 3

Summary of Efficiencies of Binding of Various Anti-LDV VP-3P Abs to Peptides of the Ectodomain of VP-3P[a]

VP-3P peptide	Specific for LDV	mAb			IMP-LDV[b]			
		159-7, 12, 18, 19	A4, E7, H9	H12	C (*T*40–*T*80)	v (*T*40–*T*80)	vx (*T*50–*T*70)	P (*T*50–*T*70)
P1	P	−	−	−	−	−	−	−
P3	P	**++++**	**++++**	**++++**	++	+++	**++++**	**++++**
P4	None	+/−	−	+/−	++	++	++	++
P5	C/v	**++++**	+++	+++	**++++**	**++++**	+++	+++
P6	vx/a	+++	+++	**++++**	++	++	**++++**	+++
P7	P	++	+++	++	+	++	++	+
P8	P	++	+++	++	+/−	+/−	+/−	−
P9	C/v	+	+/−	+/−	++	++	+/−	−
P10	P	+/−	+/−	−	−	+/−	−	−
P11	C/v	−	−	−	−	−	−	−
P12	P	+/−	+	−	−	+/−	+/−	−

[a] ++++, +++, ++, + denote decreasing binding to peptide relative to that exhibiting maximum binding **++++**; +++, ++, + indicate that the linear part of the Ab binding curve was displaced by 1–2, 2–4, or 4–6 twofold dilutions, respectively, toward the *Y*-axis (e.g., see Fig. 1). +/−, very little specific binding; −, no significant binding, i.e., comparable to that of normal mouse plasma.

[b] Summary of results from at least five individual FVB or C57BL/6 mice whose plasma (IMP) was obtained during the indicated time range p.i. (*T,* time in days) with the stated LDV quasispecies. Because of the higher levels of Abs in the plasma of the mice infected with LDV-C and LDV-v than with LDV-P or LDV-vx (Fig. 2), more alkaline phosphatase-conjugated anti-mouse IgG bound to the wells containing the same plasma dilutions of the former than the latter so that the alkaline phosphatase reaction rates differed greatly. To allow direct comparison, I have recorded the A_{405} of the ELISA plates at 25, 40, and 60 min for assessing the binding of Abs from LDV-C/LDV-v, LDV-vx, and LDV-P infected mice, respectively.

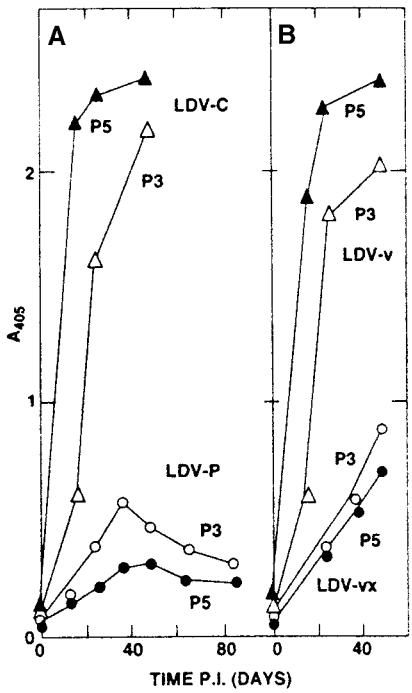

FIG. 2. Time courses of formation of P3 and P5 peptide binding Abs in LDV-C and LDV-P (A) and LDV-v and LDV-vx (B) infected mice. Plasma samples obtained at the indicated times p.i. (= *T* in days) were assayed for peptide binding by indirect ELISA as in Fig. 1. The data in A and B were obtained in single ELISAs under identical conditions, but only the A_{405} values for the 1:200 plasma dilutions are plotted.

to 4 weeks postinfection (p.i.), whereas the generation of such Abs was very slow and inefficient in mice infected with a mixture of LDV-P and LDV-vx (Plagemann *et al.,* 1999). I have now directly compared the time courses of generation of peptide binding Abs in mice infected with each of the four cloned LDV quasispecies. Figures 2A and 2B compare representative time courses of the generation of P3/P5 binding Abs in LDV-C, LDV-v, LDV-P, and LDV-vx infected mice as determined under the same experimental conditions. Comparative results were obtained with at least five mice for each LDV quasispecies. The results illustrate the rapidity and high magnitude of generation of P3/P5 binding Abs in LDV-C and LDV-v infected mice, as compared to the slow formation and much lower titers of such Abs in LDV-P and LDV-vx infected mice. The generation of these Abs was especially inefficient in LDV-P infected mice, much more so than in LDV-vx infected mice (Figs. 2A and 2B). Furthermore, it became apparent that the Abs generated in LDV-C and LDV-v infected mice always bound more efficiently to P5 than P3, whereas the opposite was the case for the Abs formed in LDV-P and LDV-vx infected mice (Figs. 2A and 2B).

Epitope specificity of peptide binding Abs generated during acute infection with various LDV quasispecies

I have compared in a single ELISA the binding of Abs in the plasma of two mice persistently infected with each of the four LDV quasispecies to P3–P12. Similar patterns were observed for the duplicate mice and for plasma samples from at least two additional mice of each quasispecies in other independent experiments. The consensus patterns are summarized in Table 3 and clearly indicated Ab binding patterns that were quasispecies specific. First, as predicted by the data in Figs. 2A and 2B, the Abs from LDV-C and LDV-v infected mice bound best to the LDV-C/v-specific peptide P5, whereas the Abs from LDV-P and LDV-vx infected mice bound best to the LDV-P-specific peptide P3. Second, the Abs from LDV-C and LDV-v infected mice bound considerably to the LDV-C/v-specific peptide P9, whereas the Abs from LDV-P and LDV-vx infected mice, like the neutralizing mAbs, bound to this peptide poorly, if at all. In addition, Abs from LDV-v infected mice bound almost as well to P3 as to P5, whereas this was never the case for Abs from LDV-C infected mice. This was surprising, since the VP-3P ectodomains of LDV-C and LDV-v are identical (Table 1). This difference suggests that additional factors may play a role in determining the specificity of the Abs that are generated in the infected mice.

Inhibition of the binding of Abs to immobilized peptides P5 and P6 by VP-3P ectodomain peptides in solution

The finding that the neutralizing mAbs and the Abs from infected mice bound only inefficiently, if at all, to the shorter VP-3P ectodomain peptides (Table 3) was unexpected since some of them must encompass the neutralizing epitope, if it is a continuous one. This failure could have been due to lack of binding of the shorter peptides to the solid phase of the ELISA wells, but this seems unlikely since peptides of similar size have been used successfully in indirect ELISAs (van Regenmortel, 1998) and most of our peptides are quite hydrophobic and were used at relatively high concentrations (up to 5 μg/well). A more likely possibility that could explain this failure is that the epitope in the longer peptides possesses some conformation required for the binding of the Abs and that this conformation is lost or that the epitope becomes sterically hindered during immobilization of the shorter peptides on the ELISA plates. I have examined this possibility by determining to what extent dilutions of VP-3P ectodomain peptides P1–P12 inhibit in solution the binding of the Abs to peptides P5 and P6 that were immobilized on the ELISA plates. For the assay I employed the highest dilution of a test Ab that did not saturate the plate-bound epitope while achieving close to maximum binding to the immobilized peptides as estimated from the indirect ELISA results (see Fig. 1). Binding competition was quantified by estimating the peptide concentrations that inhibited Ab binding to immobilized P5 or P6 by 50% (IC_{50}). The strategy is illustrated by the data in Fig. 3 for mAbs 159-18 and 159-19. Mean IC_{50} values calculated from two or more determi-

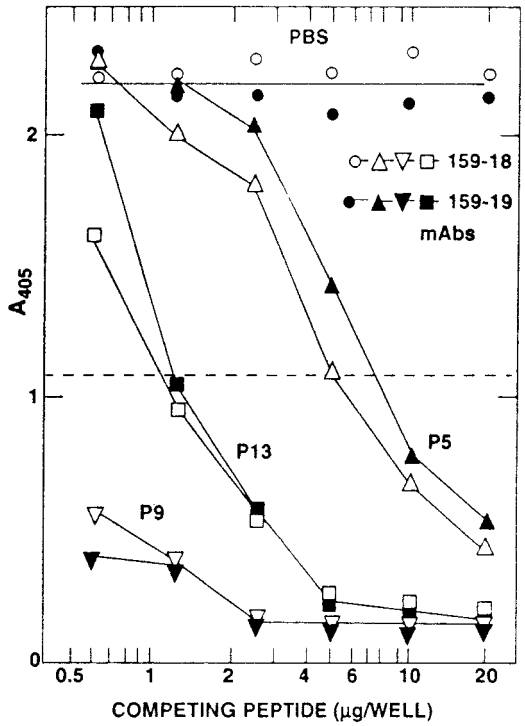

FIG. 3. Inhibition by peptides P5, P9, and P13 of the binding of mAbs 159-18 and 159-19 to immobilized peptide P5. ELISA plates coated with P5 (2 μg/well) and then incubated with blocking solution were further incubated with mixtures of 50 μl of PBS or twofold dilutions of the indicated peptides in PBS and 50 μl of a 1:20,000 dilution of mAb 159-18 or a 1:10,000 dilution of mAb 159-19 in PBS per well for 1.5 h at 37°C. The plates were then incubated with alkaline phosphatase-conjugated anti-mouse IgG and finally with substrate. The broken line indicates 50% of mAb binding in the absence of competing peptides.

nations are recorded in Table 4. All peptides tested, except the N-terminal P1 and the mutant P4, inhibited in solution the binding of the neutralizing mAbs to immobilized P5 (Table 4) and similarly to immobilized P6 (data not shown). That the binding competition by these peptides was specific was indicated by the failure of P1 and P4 to compete.

However, the degree of inhibition differed for various peptides and different mAbs. For example, P11 strongly inhibited the binding of mAbs 159-18/19 and H9/A4 to immobilized P5 and P6, whereas P11 inhibited only weakly the binding of mAb H12 (Table 4). The patterns of inhibition of the binding of the mAbs to immobilized P5 and P6 by the truncated peptides P7 to P12 in solution allowed defining the segment in the VP-3P ectodomain that was recognized by these mAbs, namely, a short segment in the center of the ectodomain, the N-terminal end of which is highly hydrophilic (Fig. 4). To verify this conclusion and to further define the epitope recognized by these mAbs I obtained an 8-amino-acid-long peptide (P13) consisting of the hydrophilic portion of the VP-3P ectodomain that is identical for all LDV quasispecies (Fig. 4) and determined its effect on the binding of the various neutralizing mAbs to immobilized P5 and P6. Indeed, P13 strongly inhibited the binding of mAbs 159-18 and 159-19 and of mAb H12 to P5 (Table 4) and P6 (data not shown). In contrast, P13 had no effect on the binding of mAb A4 and H9, whereas all other truncated peptides (P7–P12) significantly inhibited the binding of these mAbs to P5 (Table 4).

The binding of the plasma Abs from mice infected with the two classes of LDV to immobilized P5 and P6 was also inhibited by the shorter peptides in solution but the patterns of inhibition differed for Abs from LDV-C and LDV-v infected mice, on the one hand, and LDV-P and LDV-vx infected mice, on the other hand. Most important was the finding that P13 inhibited the binding of the Abs from LDV-P and LDV-vx infected mice, but affected little, if at all, the binding of Abs from LDV-C and LDV-v infected mice (Table 4). The results indicate that peptide P13

TABLE 4

Summary of Inhibition of Binding of Various Anti-LDV VP-3P Abs to Immobilized P5 by VP-3P Peptides in Solution[a]

Binding Ab		IC_{50} (μg/well) of competing peptide											
		P1	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
MAb	159-18/19	N[b]	2.5	N	6	5	2	2	<0.5	2	1	4	1.5
	H12	N	10	N	10	10	10	10	3	5	≥30	10	5
	H9/A4	N	3.5	N	4	4	6	2	4	3	1	3	N
IMP	LDV-P	N	3.5	20	5	7	7	3.5	2	3.5	8	≥40	0.5
	LDV-vx	N	5	20	5	5	5	5	1	5	5	10	0.5
	LDV-C	N	10	N	5	≥40	≥40	N	<0.5	12	10	12	≥40
	LDV-v	N	20	N	10	≥40	≥40	N	1	6	10	≥40	N

[a] ELISA plates coated with P5 or P6 (1 μg/well) were incubated with blocking solution and then with mixtures of 50 μl of PBS or twofold dilutions of the indicated competing peptides (20 or 10 to 0.6 or 0.3 μg/well) and 50 μl of test Ab (1:20,000 of mAb 159-18; 1:10,000 of mAb 159-19; 1:5000 of mAb H12; 1:1000 of mAbs A4 and H9; 1:500 of plasma from 6- to 7-week LDV-C or LDV-v infected mice; 1:100 of plasma from 7- to 8-week LDV-vx infected mice; or 1:50 of plasma from 7- to 8-week LDV-infected mice). The plates were then sequentially incubated with alkaline phosphatase-conjugated anti-mouse IgG and substrate. The presented IC_{50} values represent the concentration of competing peptide that inhibited the binding of the test Ab to immobilized P5 or P6 by 50% (e.g., see Fig. 3) and are means of two or more determinations.

[b] N, no significant competition observed in the test range of concentrations.

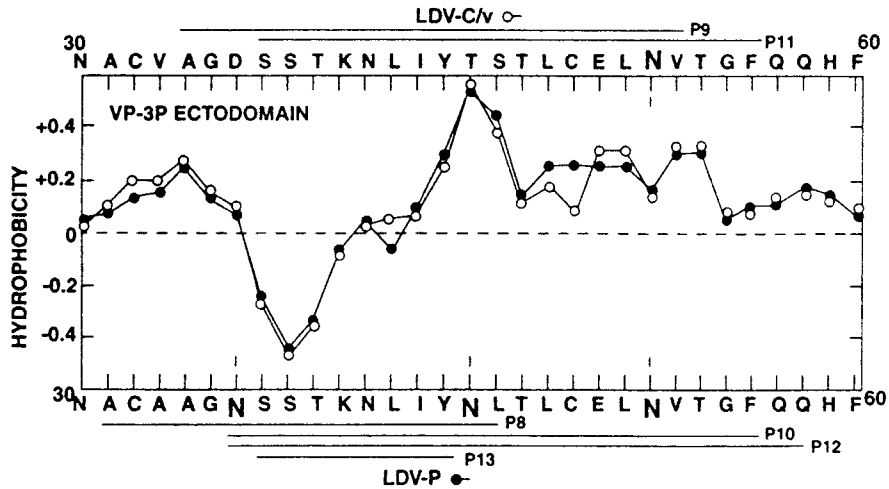

FIG. 4. Hydrophobic moment analysis according to Eisenberg *et al.* (1984) of the postulated ectodomains of VP-3P of LDV-P and LDV-C and outlines of peptides P8 to P13. The N-glycosylation sites are shown by boldface letters.

encompasses the epitope in the VP-3P ectodomain that is recognized by mAbs 159-18, -19, and H12 and Abs generated in LDV-P and LDV-vx infected mice at a low level, but that the epitope(s) recognized by mAbs A4 and H9 and the Abs generated in LDV-C and LDV-v infected mice seems to differ slightly (see Discussion).

Relationship between peptide binding Abs and Abs that neutralize the infectivity of LDV *in vitro* and *in vivo*

There exists a clear correlation between the ability of the two sets of mAbs to neutralize the infectivity of all four LDV quasispecies *in vitro* and of LDV-C/LDV-v *in vivo* and their binding to the VP-3P ectodomain peptides in an indirect ELISA (Li *et al.,* 1998; Chen *et al.,* 1999).

The same properties pertain to the VP-3P ectodomain-specific peptide binding Abs that are rapidly generated in LDV-C and LDV-v infected mice (see Fig. 2). Their appearance correlated with the formation of Abs that neutralized LDV-C and LDV-v *in vitro* and *in vivo* (Fig. 5A). Effective *in vivo* neutralization was indicated by the rapid suppression of LDV-C and LDV-v viremia in infected mice that correlated with the appearance of Abs that neutralized these LDVs *in vitro* (Fig. 5A) as well as by the finding that LDV-C and LDV-v replication is not suppressed in SCID, nude, or B-cell-deficient mice or mice that have been tolerized by infection as newborns (Chen *et al.,* 1999; Rowland *et al.,* 1994). In mice incapable of generating anti-LDV Abs, LDV-C and LDV-v establish high viremic persistent infections just like LDV-P and LDV-vx (Chen *et al.,* 1999; and unpublished data; see Fig. 5B).

In contrast, though the mAbs neutralize LDV-P/LDV-vx *in vitro,* they have no effect on the replication of these LDVs in mice (Harty and Plagemann, 1988) and the peptide binding Abs generated early in LDV-C/LDV-v infected mice that effectively neutralize these LDVs neutralize LDV-P/LDV-vx neither *in vitro* or *in vivo* (Chen *et al.,* 1999). Earlier studies have shown that Abs that neutralize LDV *in vitro* become generated in mice infected with the original isolates of LDV, LDV-PLA, LDV-ROW, LDV-NOT, and LDV-RIL (all of which were found to be mixtures of LDV-P and LDV-vx; Chen and Plagemann, 1997), begin-

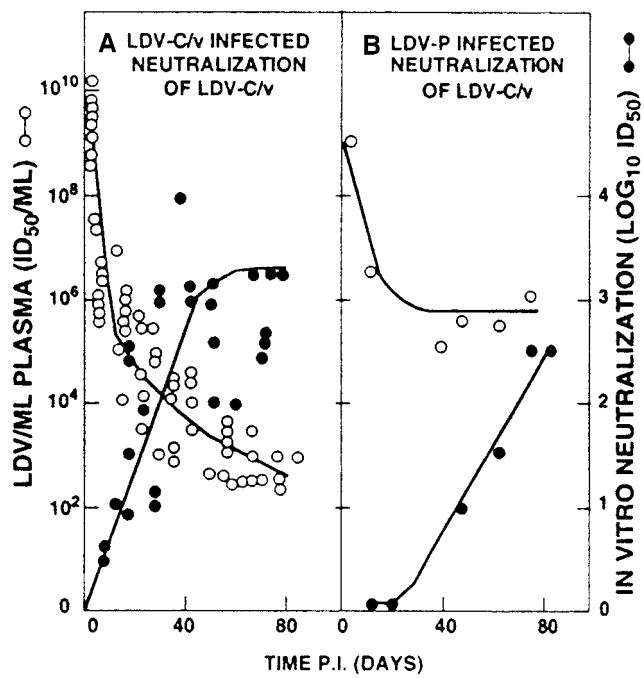

FIG. 5. Time courses of viremia and of the formation of Abs that neutralize LDV-C/v *in vitro* in mice infected with LDV-C or LDV-v (A) or cloned LDV-P (B). The data in A are combined from seven groups of two FVB or C57BL/6 mice monitored in independent experiments (also see Chen *et al.,* 1999; Plagemann *et al.,* 1999). The data in B are for plasma pooled from two FVB mice. In each case, plasma was obtained at the indicated time p.i. and assayed for infectious LDV by an end-point dilution assay in mice (○-○) and for *in vitro* neutralization of LDV-C or LDV-v (●-●) and selected samples were analyzed by RT-PCR using quasispecies-specific primers to confirm the presence of the individual quasispecies used for infection.

ning about 1 month p.i., but only at a low level and without affecting viremia (Rowson and Mahy, 1975; Plagemann, 1996). I have now confirmed these results using biologically cloned LDV-P. Abs that neutralized LDV-C (and LDV-P) *in vitro* became generated beginning at about 1 month p.i. (Fig. 5B), and, although these Abs were effective at neutralizing these LDVs *in vitro,* their appearance had no effect on LDV-P viremia (Fig. 5B). More importantly, there was no correlation between the time courses and levels of generation of Abs that neutralized LDV *in vitro* and those that bound to the various VP-3P ectodomain-specific peptides (cf. Figs. 2A and 5B). In fact, the generation of *in vitro* neutralizing Abs occurred largely in the absence of formation of peptide binding Abs. Thus most of the late Abs that neutralize LDV-P *in vitro* seem to bind to an epitope distinct from the linear VP-3P ectodomain epitope, more likely a discontinuous epitope (see below). Abs to the same or a similar epitope also seem to become generated in LDV-C and LDV-v infected mice at about 1 month p.i. since at this time Abs appear that neutralize LDV-P and LDV-vx *in vitro* (Chen *et al.,* 1999).

Properties of anti-peptide Abs

I have immunized mice with the LDV-P-specific P3 peptide and the LDV-C/v-specific P5 peptide that were conjugated via their Cys residue (see Table 1) to bovine serum albumin (BSA). The mice developed very high titers of Abs (1:25,000; data not shown) that bound in the indirect ELISA to both immobilized P3 and P5, but almost as well to the mutated P4. The anti-peptide Abs also bound in an LDV quasispecies-specific manner to the truncated peptides that are not recognized in the indirect ELISA by the neutralizing mAbs or Abs from infected mice. The anti-P3-BSA Abs bound significantly to the LDV-P-specific peptides P7, P10, and P12, whereas the anti-P5-BSA Abs bound to the LDV-C/v-specific P9 peptide. Furthermore, none of the peptides, except P5, competed in solution with the binding of either the anti-P3-BSA or the anti-P5-BSA Abs (at a 1:5000 dilution) to immobilized P5 (data not shown). The Abs also lacked neutralizing activity. The replication of LDV-C and LDV-P was the same in the peptide-immunized mice as in nonimmunized mice and the Abs failed to neutralize these LDVs *in vitro* (data not shown). Together these results indicate that the anti-peptide Abs recognize denatured forms of the LDV-P and LDV-C/v neutralizing epitopes and that only Abs to the native epitopes are neutralizing.

DISCUSSION

Altogether, my results indicate that the neutralizing mAbs and the peptide binding Abs generated at a low level in LDV-P and LDV-vx infected mice recognize a linear epitope in an 8-amino-acid-long segment (P13) located in the hydrophilic center of the VP-3P ectodomain (Fig. 4). This location of the neutralization epitope now explains why the N-terminal and the middle N-glycans on the VP-3P ectodomain are those that impair the immunogenicity of the epitope and are responsible for the resistance of LDV-P and LDV-vx to Ab neutralization. They are flanking the neutralization epitope (Fig. 4) and their loss, as in LDV-C and LDV-v, is sufficient to render the epitope highly immunogenic (Figs. 2A and 2B) and the virus sensitive to Ab neutralization (Fig. 5A). Also, neutralization escape mutants of LDV-C and LDV-v have been shown to have regained the middle N-glycosylation site and an N-terminal N-glycosylation site (Chen *et al.,* 2000). The C-terminal N-glycan (see Fig. 4) does not seem to have any effect on the immunogenicity of the epitope because it is located about 8 residues downstream of it and passed the ^{49}Cys residue thought to form the S-S bond that links the ectodomain of VP-3P to the even shorter (about 11 amino acids long) ectodomain of the M protein (Faaberg *et al.,* 1995). This N-glycosylation site is preserved in all LDV isolates whether sensitive or resistant to the humoral immune response and also on the ectodomains of all isolates of the related arterivirus, porcine reproductive and respiratory syndrome virus (Chen *et al.,* 2000), and thus probably plays some other vital function in virus replication. On the other hand, all three N-glycans may participate in the polyclonal activation of B cells caused specifically by LDV-P/LDV-vx infection (Plagemann *et al.,* 2000). The low immunogenicity of the linear neutralization epitope of LDV-P and LDV-vx may explain why neutralizing Abs became elicited in a rabbit only after numerous injections of semipurified LDV-P/LDV-vx (Cafruny and Plagemann, 1982) recognizing another low-immunogenicity epitope on VP-3P (the Abs do not bind to any of the synthetic peptides; unpublished data). The low immunogenicity of the neutralization epitope of LDV-P and LDV-vx is confined to this epitope; Abs to other epitopes on VP-3P and to other viral proteins become generated in infected mice as rapidly and efficiently as the neutralization Abs in LDV-C and LDV-v infected mice (Cafruny *et al.,* 1986; Plagemann *et al.,* 1995). These immune responses are associated with the appearance in the spleen and lymph nodes of germinal centers that contain large numbers of virions most likely trapped by follicular dendritic cells (Plagemann *et al.,* 1995).

The epitope recognized by the neutralizing Abs elicited in LDV-C and LDV-v infected mice seems to have shifted slightly downstream since the binding of the Abs to immobilized P5, though not inhibited by P7, P8, and P13, is inhibited by the peptides that extend further downstream, P9–P12, especially the LDV-C/v-specific P9 (Table 4). The Abs from LDV-C/LDV-v infected mice also bind directly to P9, while poorly binding to P8 located further upstream (Table 3). This epitope shift is probably the result of amino acid substitutions associated with changes in the hydrophobicity profile of the ectodomain (Fig. 4).

Conformation of the linear epitope of the VP-3P ectodomain in LDV virions plays an important role in the ability of the virions to induce the generation of Abs that neutralize LDV and bind to the epitope containing peptides in ELISAs. The Abs generated in infected mice as well as the neutralizing mAbs bound best to the full-length ectodomain peptides (P3, P5, P6) and only poorly, if at all, to most of the shorter truncated peptides (Table 3). This lack of binding to the shorter peptides seems to have been due to a change in conformation or generation of steric hindrance during immobilization in the ELISA wells since the same peptides in solution efficiently blocked the binding of the Abs to the immobilized full-length peptides (Table 4). Furthermore, it is clear that the peptide binding Abs generated in mice infected with the four different LDV quasispecies exhibit quasispecies-specific differences in binding to the various peptides (Tables 3 and 4), which must be due to amino acid differences outside the epitope(s), most likely affecting their conformation. In addition to the structure of the VP-3P ectodomain per se, factors outside the ectodomain, such as perhaps its linkage to the M protein, can also affect the conformation of the neutralizing epitopes, since the peptide binding patterns of the Abs raised to LDV-C and LDV-v differed slightly (Table 4), even though the amino acid sequence of the VP-3P ectodomains of the two LDVs is identical (Table 1).

The importance of epitope conformation was also indicated by the failure of the BSA-conjugated full-length peptides to induce Abs in mice that neutralized LDV, even though the peptides were linked to BSA via the same Cys residue that is thought to be involved in the linkage of the VP-3P ectodomain to the ectodomain of the M protein. The BSA–peptide conjugates induced high levels of Abs that bound in the indirect ELISA to P3, P4, and P5, but apparently to a denatured form of the neutralization epitope, since they bound in an LDV quasispecies-specific manner to the shorter peptides when immobilized, but failed in solution to block the binding of the Abs to immobilized P5. Thus only Abs to the native form of the linear VP-3P ectodomain epitope possessed neutralizing activity. This finding is of some interest in relation to the use of peptides in antiviral vaccines. In the case of another arterivirus, equine arteritis virus, vaccination with a conjugate of keyhole limpet hemocyanin and a peptide containing a neutralization epitope from the ectodomain of the primary envelope glycoprotein induced neutralizing Abs in horses but not in a rabbit (Chirnside *et al.,* 1995).

The conformation or nature of the neutralization epitope also seems to be affected by various treatments used to inactivate virions for vaccination since the peptide binding patterns of the sets of neutralizing mAbs generated to formalin-inactivated and UV-light-inactivated LDV-P/LDV-vx differ slightly from that of the Abs from LDV-P/LDV-vx infected mice and from each other (Tables 3 and 4), but the nature of the alteration is unknown. Nevertheless, the binding of the mAbs to the neutralization epitope correlates with their virion neutralization ability (Li *et al.,* 1998; Chen *et al.,* 1999). In contrast, glutaraldehyde inactivation of virions appears to destroy the epitope since the mAbs generated to glutaraldehyde-inactivated LDV-P/LDV-vx, though reacting with VP-3P in immunoblots, failed to neutralize LDV-P/LDV-vx (Harty *et al.,* 1987) and to bind to P3 and P5 in the indirect ELISA (data not shown).

The results are also of interest in that they detected the generation of a second type of Ab that neutralizes LDV *in vitro.* These Abs become generated in LDV-P/LDV-vx as well as LDV-C/LDV-v infected mice, but only beginning about 1 month p.i. and at low levels. These Abs do not bind significantly to the peptides containing the linear neutralizing epitope in the VP-3P ectodomain, but instead are generated to another VP-3P epitope that is weakly immunogenic and may also be recognized by the rabbit neutralizing Abs (see above). The nature of this epitope is unclear, but it is most likely discontinuous and formed by the linkage of the VP-3P ectodomain to that of the M protein. Abs to this epitope neutralize LDV-P/LDV-vx and LDV-C/LDV-v *in vitro* to about the same extent (Chen *et al.,* 2000), but only inefficiently requiring the binding of many Ab molecules per virion (Cafruny *et al.,* 1986; Chen *et al.,* 1999; Plagemann *et al.,* 1992). These Abs fail to affect LDV-P and LDV-vx replication in mice (Fig. 5B), probably because they never attain a high enough level *in vivo* to mediate efficient virion neutralization. During the persistent phase of infection practically all virions are present in the circulation as infectious Ab–virus complexes (Cafruny *et al.,* 1986). Combined, this virion sequestration by Abs and the blockage of the linear neutralization epitope on the VP-3P ectodomain by N-glycans allow continuous cycles of cytopathic replication of LDV-P and LDV-vx in a renewable subpopulation of tissue macrophages that supports the lifelong persistent infection. It seems likely that other viruses have developed this type of mechanism to escape the humoral immune response of the host.

MATERIALS AND METHODS

Mice

FVB mice were provided by the transgenic facilities of the University of Minnesota. C57BL/6 mice were purchased from The Jackson Laboratories. Plasma was obtained from mice by the orbital bleeding method using heparinized blood collection tubes (Fisher Scientific, Pittsburgh, PA) as described previously (Chen and Plagemann, 1997).

LDVs

The common nonneuropathogenic LDV-P and LDV-vx have been previously cloned by end-point dilution in mice from the original LDV isolates LDV-PLA and LDV-

VIR, respectively, and the neuropathogenic laboratory mutants LDV-C and LDV-v were similarly cloned from the original neuropathogenic isolates LDV-C-BR and LDV-VIR, respectively (Chen *et al.,* 1998, 1999; Chen and Plagemann, 1997).

LDV concentrations were estimated by an end-point dilution assay in FVB mice (Chen and Plagemann, 1997). The assay is based on the 5- to 10-fold increase in plasma lactate dehydrogenase activity invariably associated with an LDV infection (Plagemann, 1996; Plagemann and Moening, 1992). LDV titers are expressed as ID_{50}. LDV stocks consisted of plasma harvested from mice 1 day p.i. and contained 10^9 to 10^{10} ID_{50}/ml. Mice were infected by intraperitoneal injection of about 10^6 ID_{50}.

Measurement of the binding of Abs to VP-3P peptides by indirect ELISA

The procedure was as described previously (Li *et al.,* 1998). In brief, the wells of 96-well ELISA plates were coated with synthetic peptides of various lengths representing the ectodomains of VP-3P of various cloned LDV quasispecies (Table 1) in carbonate buffer, pH 9.6 (2 μg/well). The plates were then sequentially incubated with blocking solution [1% (w/v) BSA and 0.25% (v/v) Tween 20 in phosphate-buffered saline (PBS)], twofold dilutions of various neutralizing mAbs (Table 2) or of plasma from infected mice, alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO), and enzyme substrate (Sigma 104). Between each incubation with the next reagent the plates were rinsed 3 or 4 times with 0.05% (v/v) Tween 20 in PBS (PBS–Tween). The absorbance of the alkaline phosphatase reaction product was measured at 405 nm (A_{405}) with an automatic microplate reader. The synthetic peptides (Table 1) were synthesized and purified as previously described (Li *et al.,* 1998) or synthesized and purified by the Microchemical Facility of the University of Minnesota.

Measurement of inhibition of Ab binding to immobilized P5 and P6 peptides by VP-3P ectodomain peptides in solution

ELISA plates were coated with ORF 5 peptides P5 or P6 (Table 1; 1 or 2 μg/well). The plates were rinsed twice with PBS–Tween and then incubated with blocking solution for 45 min at 37°C followed by incubation for 1.5 to 2 h at 37°C with mixtures of 50 μl of an Ab solution and 50 μl of twofold dilutions of VP-3P ectodomain peptides (Table 1) in PBS generally from 20 or 10 to 0.6 or 0.3 μg/well. The plates were rinsed thrice with PBS–Tween, sequentially incubated with alkaline phosphatase-conjugated anti-mouse IgG and substrate, and finally read at 405 nm as already described. The solutions of specific Abs used in the test were the highest dilutions of Ab that elicited maximum binding to immobilized peptides P3 and/or P5 as determined by indirect ELISA (see Figs. 1 and 3). The Ab dilutions used are stated in the footnote to Table 4.

Measurement of *in vitro* Ab neutralization of LDV

As described previously (Chen *et al.,* 1999; Plagemann *et al.,* 1992), samples of LDV stocks were incubated *in vitro* with undiluted normal mouse plasma (NMP) or plasma from LDV infected mice (IMP). The residual LDV infectivity in both samples was determined by titration in mice. The degree of neutralization was defined as the difference in titer between the LDV samples incubated with NMP and IMP and was expressed in $\log_{10}$ ID_{50}.

Immunization of FVB mice with peptides P3 and P5

Peptide–BSA conjugates were produced essentially as described by Maloy *et al.* (1994). In brief, 1 ml of BSA in 0.01 M phosphate buffer, pH 7.1 (10 mg/ml), was mixed with 0.2 ml *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester in dimethylformamide (10 mg/ml). The mixture was incubated at room temperature for 30 min with stirring and the activated BSA isolated by Sephadex G25 chromatography. After the pH of the eluate was adjusted to 7.0, 1.3 ml was mixed with 0.5 ml of 6 M guanidine hydrochloride containing about 2 mg peptide P3 or P5 per milliliter. The mixtures were incubated at room temperature for 3 h and then dialyzed at 4°C once against 2 liters dH_2O overnight, twice against 2 liters dH_2O for 3 h, and once against 2 liters PBS for 3 h. A sample of the final conjugate solution containing about 1.5 mg protein/ml was vigorously mixed with an equal volume of CFA and 0.2 ml of the mixture was injected intraperitoneally into each of three mice. At 7 and 31 days later the mice were reinoculated with a similar mixture of conjugate and IFA. Plasma was obtained 7 days after the last injection and assayed for peptide binding activity. At this time individual mice immunized with P3–BSA or P5–BSA and a nonimmunized mouse were injected with 10^6 ID_{50} of LDV-C, LDV-v, or LDV-P. The mice were bled at intervals thereafter and their plasma was analyzed for infectious LDV and peptide binding Abs.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from plasma of infected mice and reverse transcribed and the cDNA was amplified by PCR using LDV-P-, LDV-vx-, and LDV-C-specific primers as described previously (Chen and Plagemann, 1997; Chen *et al.,* 1998, 1999).

ACKNOWLEDGMENTS

I thank Ron Jemmerson for advice and editorial comments, J.-P. Coutelier for providing anti-LDV mAbs, and Jodi Rucker for excellent secretarial assistance.

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