JOURNAL OF VIROLOGY, Sept. 1988, p. 3210–3216
0022-538X/88/093210-07$02.00/0

Vol. 62, No. 9

Formalin Inactivation of the Lactate Dehydrogenase-Elevating Virus Reveals a Major Neutralizing Epitope Not Recognized during Natural Infection

JOHN T. HARTY AND PETER G. W. PLAGEMANN*

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455-0312

Received 29 February 1988/Accepted 2 June 1988

Five hybridomas that secrete monoclonal antibodies which neutralize the infectivity of lactate dehydrogenase-elevating virus (LDV) were isolated from BALB/c mice primed with Formalin-inactivated LDV. Competition analyses indicated that all five neutralizing monoclonal antibodies recognize contiguous, if not identical, epitopes on the envelope glycoprotein of LDV (VP-3) which are not recognized by nonneutralizing VP-3-specific monoclonal antibodies isolated from the same fusion. Despite the presence of neutralizing activity, polyclonal anti-LDV antibodies obtained from persistently infected mice did not compete for binding to LDV with four of the five neutralizing monoclonal antibodies tested. The results indicate that the envelope glycoprotein of LDV possesses a major neutralizing epitope which is poorly recognized, if at all, by mice during a natural infection but is rendered immunogenic by Formalin inactivation of the virus. The epitope was also not immunogenic in a rabbit, since its polyclonal LDV-neutralizing antibodies did not inhibit binding of the mouse monoclonal antibodies to LDV. Passive immunization with the neutralizing monoclonal antibodies did not protect mice from LDV infection and did not alter the course of infection. Neutralizing monoclonal antibodies have been used to select a neutralization escape variant by a novel combination of in vitro and in vivo isolation.

Lactate dehydrogenase-elevating virus (LDV) is a murine togavirus which causes a lifelong persistent infection that is usually not associated with disease manifestations (34). Persistence is maintained by replication of LDV in macrophages (36, 37), despite the presence of a strong humoral immune response directed predominantly toward the viral envelope glycoprotein VP-3 (4, 9). Although high levels of anti-LDV antibodies are generated in persistently infected animals, antibodies which neutralize LDV infectivity are not detectable until 1 to 2 months postinfection (p.i.). It appears that these neutralizing antibodies do not constitute a major fraction of the humoral response to LDV, since only low levels of neutralizing activity are observed in polyclonal antisera (7, 29, 30, 34). The reason for the poor neutralizing antibody response to LDV and the relationship between the inability of polyclonal antisera to neutralize LDV efficiently and the ability of the virus to persist are not clear. One possibility could involve the inability of mice to recognize an essential neutralizing epitope(s) on the glycoprotein of LDV. Another possibility which may account for the poor neutralizing response to LDV is suggested by the observation that during the persistent phase of infection essentially all LDV is present in infectious virus-antibody complexes (6, 30). Thus, blocking antibodies may prevent recognition or binding (or both) of the essential neutralizing epitope(s) on LDV.

Hybridoma technology has proven valuable in the study of virus neutralization, particularly with respect to identification of critical target sites (epitopes) and potential mechanisms of neutralization (10, 11, 14, 24, 27, 35). Previously, we (18) and other investigators (9) found that fusion with spleens from LDV-infected mice yielded anti-LDV monoclonal antibody (MAb)-producing hybridomas with very low frequency. For example, in our study only 1 of 297 hybridomas made with spleens from infected mice produced an LDV-specific MAb (18). This MAb is specific for the envelope glycoprotein of LDV, VP-3, but fails to neutralize the infectivity of the virus. In contrast, when mice were immunized with glutaraldehyde-inactivated LDV before infection, over 25% of the resulting hybridomas were LDV specific. However, none of 12 MAbs that we analyzed neutralized LDV infectivity, in spite of being specific for VP-3 and expressing immunoglobulin M (IgM), IgG1, IgG2a, and IgG2b isotypes. We now report the isolation of hybridomas that secrete neutralizing MAbs after mice are primed with Formalin-inactivated LDV, and we discuss reasons why mice fail to recognize the epitope(s) identified by these neutralizing antibodies in persistent infection.

MATERIALS AND METHODS

Animals. Female BALB/c mice were bred in the animal facility of the Department of Microbiology, University of Minnesota, Minneapolis. Swiss mice used in LDV titrations were obtained from BioLabs, Inc., St. Paul, Minn.

Virus. Groups of 50 to 150 Swiss mice were infected with the strain of LDV originally isolated in this laboratory (LDV_{PLA}; 3) or a strain obtained from W. Murphy (LDV_{MUR}; 28), and their plasma was harvested at 1 day p.i. LDV was purified from the plasma by isopycnic centrifugation in sucrose density gradients (7). LDV concentrations were determined by an endpoint dilution assay in mice, as described previously, and expressed as 50% infective doses (ID_{50}) (32).

Isolation of anti-LDV hybridomas. Hybridomas that secrete MAbs to LDV_{PLA} were isolated as previously described (18), except that LDV was inactivated by incubation in 0.06% (vol/vol) Formalin at 37°C for 5 h. Complete inactivation was ascertained by inoculation into mice. Inactivated virus was emulsified in complete Freund adjuvant and injected subcutaneously into female BALB/c mice. At 14 days after the initial immunization, the same animals were inoculated with inactivated LDV emulsified in incomplete Freund adjuvant. Animals were infected with infectious

* Corresponding author.

LDV 36 days later, when a significant anti-LDV response in the immunized animals had developed. The purpose of infecting animals before use of their spleen cells in fusions was to increase the likelihood of isolating clones reactive with native virus and to stimulate a general activation of B cells (6, 18). At 4 days p.i., spleen cells were fused with NS-1 cells, the resulting hybridomas were screened and subcloned, and ascites fluid was prepared in BALB/c mice as previously described (18).

Anti-LDV IgG determinations. Anti-LDV antibodies were quantitated by a fluorescent-antibody (FA) staining assay as described previously (4). In brief, primary macrophage cover slip cultures were infected with LDV and fixed in acetone at 8 h p.i. Replicate fixed cultures were sequentially incubated with twofold serial dilutions of anti-LDV antibodies and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG. The FA titer was expressed as the reciprocal of the highest antibody dilution that yielded recognizable staining of 3 to 10% of the total macrophages in LDV-infected cultures without staining any cells in uninfected cultures.

LDV neutralization assay. Plasma from 1-day infected mice (about 10^9 ID_{50}/ml), which is devoid of anti-LDV antibodies (4), was diluted 1:100 in phosphate-buffered saline (PBS), and 10-μl samples thereof were mixed with 20 μl of normal mouse plasma, plasma from infected mice, ascites fluid containing anti-LDV MAbs, or normal or anti-LDV immune rabbit plasma diluted 1:10 (7). The mixtures were sequentially incubated at 37°C for 3 to 4 h and at 4°C for 1 to 2 h and then analyzed for infectious LDV. The degree of neutralization was defined as the difference between LDV samples incubated with normal plasma and those incubated with antibody preparations. Analysis of the ability of a MAb to neutralize LDV obtained from persistently infected mice that had developed an anti-LDV response was performed as described above, except that the virus was harvested from groups of BALB/c mice at various times (4 to 107 days) p.i. and the plasma was diluted only 1:10 with PBS before being mixed with the antibody.

Labeling of MAbs with HRP. MAbs were purified from ascites fluid by protein A–Sepharose CL-4B (Pharmacia) affinity chromatography essentially as described by Ey et al. (13). Purified MAbs were coupled to horseradish peroxidase (HRP) by a two-step glutaraldehyde procedure as described by Engvall (12). Antibody-HRP conjugates were stored in the dark at 4°C in 50% (vol/vol) glycerol.

Competitive ELISA. A sample (50 μl) of a 1:50 dilution of purified LDV_{PLA} (~10^9 ID_{50}/ml) in PBS (pH 7.4) was added to each well of a 96-well flat-bottom microtiter plate (Linbro 76-381-04). After incubation overnight at 4°C, the plates were incubated for 1 h at room temperature with PBS containing 1% (wt/vol) bovine serum albumin to block nonspecific protein binding. After washing the plates with PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween), 100-μl serial dilutions of MAb-containing ascites fluid in PBS-Tween were added to the appropriate wells. The plates were incubated for 3 h at room temperature with rocking, followed by washing with PBS-Tween. HRP-conjugated MAbs diluted with PBS-Tween were added to the appropriate wells, and the plates were incubated for 2.5 h at room temperature with rocking. After being washed with PBS-Tween, the plates were developed with H_2O_2 and *o*-phenylenediamine as previously described (12). The reactions were stopped by addition of 4 N H_2SO_4, and the A_{492} was read. Competition was evaluated by comparison with a control ascites fluid (2653A, containing a MAb to *Myxococcus xanthus*, supplied by M. Dworkin).

TABLE 1. Characteristics of anti-LDV MAbs from mice primed with Formalin-inactivated virus[a]

Clone	Isotype	FA titer[b]	Neutralization[c]
159-3	IgG1	>15,000	−
159-4	IgG1	>10,000	−
159-5	IgG1	>15,000	−
159-7	IgG2a	>15,000	+
159-12	IgG2b	>15,000	+
159-13	IgG1	>10,000	−
159-14	IgG1	>12,000	−
159-16	IgG2b	8,000	+
159-18	IgG2a	>15,000	+
159-19	IgG1	8,000	+

[a] All of the MAbs were specific for VP-3 as determined by Western blot analysis (Fig. 2).

[b] The FA titer of ascites fluid was determined as described in Materials and Methods.

[c] Undiluted ascites fluid neutralized ≥1.5 $\log_{10}$ ID_{50} of LDV under the conditions described in Materials and Methods.

Isolation of neutralization escape variant. The LDV neutralization assay was performed as already described. After titration, animals inoculated with the highest dilution that resulted in LDV infection after treatment with MAbs were bled and their plasma was inoculated into Swiss mice to amplify the virus for further selection. This procedure was repeated until a variant resistant to neutralization by the MAb was isolated.

RESULTS

Isolation and characterization of neutralizing MAbs. In contrast to our previous report describing the isolation of nonneutralizing anti-LDV MAbs from mice primed with a glutaraldehyde-inactivated virus (18), 5 of 10 VP-3-specific hybridomas derived from mice primed with Formalin-inactivated LDV exhibited considerable neutralizing activity (Table 1). Neutralizing activity was assessed by incubating LDV harvested from 1-day infected mice with MAbs in vitro and then measuring residual infectivity by titration in mice (see Materials and Methods). Under these conditions, undiluted ascites fluid of MAbs 159-7, 159-12, 159-16, 159-18, and 159-19 (FA titers of 8,000 to >15,000 [Table 1]) consistently neutralized 1.5 $\log_{10}$ to 3 $\log_{10}$ LDV. The neutralizing activity was comparable to or higher than that of polyclonal anti-LDV in plasma of 2- to 3-month LDV-infected mice (immune mouse plasma [IMP]; e.g., see Fig. 1). Although the MAbs were raised to the LDV_{PLA} strain, they were equally effective in neutralization of the LDV_{MUR} strain (data not shown), which exhibits greater neurovirulence in C58 mice (23, 28). This type of neutralizing response is similar to that seen with polyclonal IMP (4) but differs from neutralization by polyclonal rabbit anti-LDV_{PLA} (immune rabbit plasma [IRP]), which is more effective in neutralizing the homologous strain of LDV than several heterologous strains of LDV (7).

Another similarity between the MAbs and IMP is the temperature dependence of neutralization (7). Effective neutralization was observed at 37°C with the MAbs and IMP (Fig. 1). However, relatively little neutralization occurred at 4°C. In contrast, polyclonal anti-LDV IRP neutralized LDV efficiently at both 37 and 4°C (7; Fig. 1). Furthermore, the neutralization titers of the MAbs (≤100; Fig. 1) were relatively low compared with the FA titers of these ascites fluids (≥8,000; Table 1). This discrepancy was also observed with polyclonal anti-LDV IMP and may reflect a similarity in the

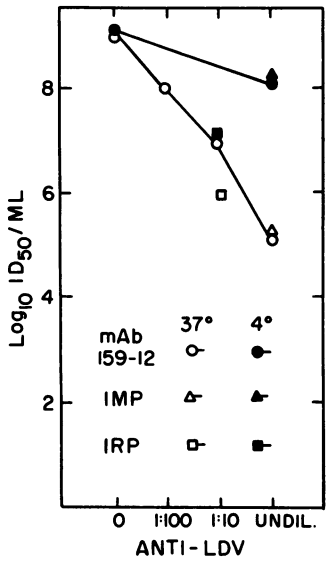

FIG. 1. Neutralization of LDV by MAb 159-12, anti-LDV IMP, or IRP. The assay was conducted as described in Materials and Methods. Samples of LDV_{PLA} were incubated for 4 h as indicated, with normal mouse plasma (0 = control) or undiluted (UNDIL.) anti-LDV and dilutions thereof at 37 and 4°C and then assayed for residual infectivity by titration in mice.

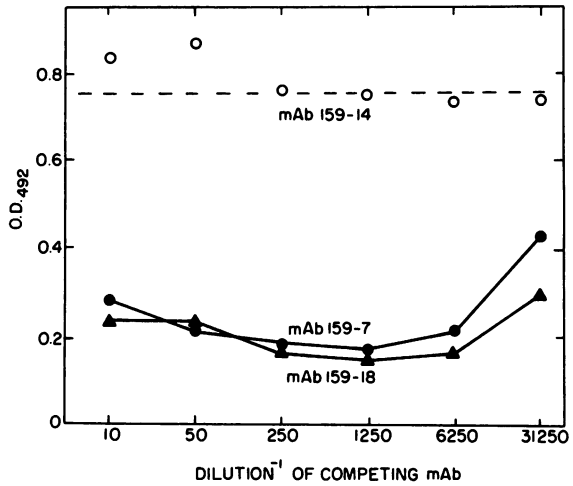

FIG. 3. Competitive ELISA between neutralizing anti-LDV MAbs. The assay was conducted as described in Materials and Methods. LDV-coated wells were incubated with 100 μl of serial fivefold dilutions of ascites fluid containing unconjugated MAb 159-7, 159-14, or 159-18 and then with HRP-conjugated MAb 159-18. All values are means of triplicate wells. The standard deviations did not exceed 10% of the mean. The broken line indicates the mean absorbancy obtained with an unrelated control MAb at a 1:10 dilution. $O.D._{492}$, Optical density at 492 nm.

mechanisms of neutralization. Representative Western blots (immunoblots) illustrating the specificities of some of the neutralizing and nonneutralizing MAbs for VP-3 are shown in Fig. 2. The pattern of reaction with VP-3, which migrates heterogeneously in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (3, 4), was similar for all

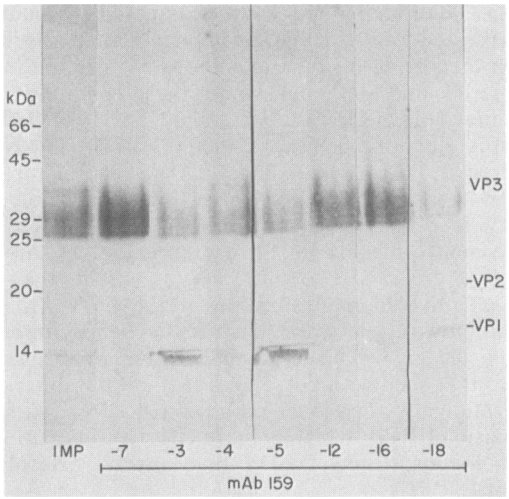

FIG. 2. Immunoblotting of LDV proteins with mouse polyclonal anti-LDV IgG and MAbs to Formalin-inactivated LDV. Samples of purified LDV_{PLA} were electrophoresed in 15% polyacrylamide-sodium dodecyl sulfate gels and immunoblotted as described previously (4). The blots were reacted as indicated, with plasma from 4-month LDV-infected mice (IMP, diluted 1:50) or ascites fluid containing the indicated MAbs (diluted 1:50), and then sequentially incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin and the appropriate substrate. The variable staining at about 14 kilodaltons (kDa) was an artifact which was linked to contamination of the LDV preparation by a host protein (4).

MAbs whether neutralizing or nonneutralizing and similar to that observed with polyclonal IMP. Furthermore, results from repeated Western blots indicated that the relative abilities of all of these MAbs to react with VP-3 were about the same.

Four of the five neutralizing MAbs were of the IgG2a and IgG2b isotypic subclasses, and one neutralizing MAb was of the IgG1 subclass, whereas all of the nonneutralizing MAbs were IgG1 (Table 1). The significance of this isotype distribution is not clear. It does not seem to reflect a greater efficiency of antibodies of the IgG2 isotypes in mediating complement-dependent lysis of LDV. Addition of guinea pig complement at a 1:10 dilution in the standard neutralization assay did not increase neutralization by two of the neutralizing MAbs (159-12 and 159-18) or cause neutralization by one of the nonneutralizing MAbs (159-13; data not shown). It has been previously shown that complement has little effect on neutralization of LDV by polyclonal anti-LDV IMP (4) and complement does not bind anti-LDV antibodies efficiently (25, 31).

Epitope mapping with neutralizing MAbs. Antibody-mediated neutralization of virus infectivity may involve a variety of mechanisms, such as physical elimination of infectious particles by complement-mediated lysis or aggregation, prevention of attachment to cellular receptors, or prevention of viral uncoating (8, 16, 21, 22, 38). As such, neutralization may require interaction of antibodies with critical epitopes as opposed to polyvalent binding or the ability to fix complement. We used solid-phase competitive binding assays with purified neutralizing MAbs conjugated with HRP as the detection system and ascites fluid as the source of the competitor MAb to determine the potential number of epitopes involved in neutralization by these MAbs. Figure 3 illustrates typical results from such a competition ELISA. Unconjugated MAbs 159-7 and 159-18 in the form of ascites fluid clearly inhibited binding of HRP-conjugated MAb 159-18 up to dilutions of >1:30,000, whereas the nonneutralizing MAb 159-14 had no effect. There was complete reciprocal

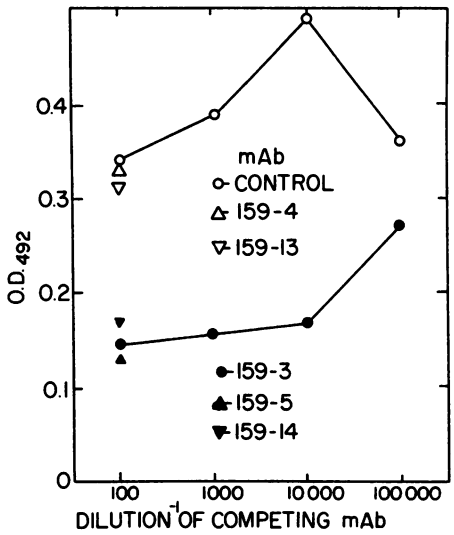

FIG. 4. Competitive ELISA between nonneutralizing MAbs. The assay was conducted as described in Materials and Methods. LDV-coated wells were incubated with serial 10-fold dilutions of ascites fluid containing MAbs 159-4, 159-5, 159-13, and 159-14, followed by HRP-conjugated MAb 159-3. All values are means of triplicate wells. The standard deviations did not exceed 10% of the mean. $O.D._{492}$, Optical density at 492 nm.

competition between the neutralizing MAbs, at least among the four MAbs that were conjugated to HRP (159-7, 159-12, 159-16, and 159-18). Each neutralizing MAb, as a 1:50 dilution of ascites fluid, decreased the binding of all four HRP-conjugated MAbs (at a dilution of 1:200) over 75%, and neutralizing MAb 5 (159-19) was equally effective in inhibiting the binding of all four HRP-conjugated MAbs to LDV. In contrast, none of the nonneutralizing MAbs had any effect. Thus, all five neutralizing MAbs recognized a contiguous, if not identical, epitope of the envelope glycoprotein of LDV, whereas the nonneutralizing MAbs recognized a different epitope(s).

We also conjugated one nonneutralizing MAb (159-3) to HRP for use in competition assays. None of the five neutralizing MAbs in the form of ascites fluid was able to compete for binding with 159-3–HRP at dilutions as low as 1:10 (data not shown). On the other hand (Fig. 4), nonneutralizing MAbs 159-5 and 159-14 did compete for binding with 159-3–HRP at a dilution of 1:100 to an extent similar to that of the homologous 159-3 ascites fluid. Nonneutralizing MAbs 159-4 and 159-13 did not compete for binding with 159-3–HRP (Fig. 4) nor did any of the MAbs isolated from mice primed with glutaraldehyde-inactivated LDV (18; data not shown). These data indicate that at least three distinct epitopes exist on the envelope glycoprotein of LDV, one of which can function in neutralization.

Epitope specificity of antibodies produced in LDV-infected mice. Neutralization by the five MAbs shares several characteristics with neutralization by polyclonal anti-LDV IMP, including temperature dependence, complement independence, and the ability to neutralize several strains of LDV which differ in neurovirulence for C58 mice. We performed competition experiments to determine whether the same epitope that is recognized by the neutralizing MAbs is responsible for generating the neutralizing response in persistently infected mice. Pools of plasma obtained from 21- and 77-day LDV-infected BALB/c mice (FA titers, >5,000; reference 4) failed to inhibit binding of neutralizing MAb 159-12–HRP, despite the presence of neutralizing activity in the IMP from 77-day infected mice (data not shown). The IMP from 77-day infected mice failed to compete with the neutralizing MAb whether or not the plasma had been extracted with ether to inactivate the LDV present in the IMP in the form of infectious antibody-virus complexes (7, 30). These data suggest that the single neutralizing epitope on the envelope glycoprotein of LDV which is recognized by the neutralizing MAbs is poorly recognized, if at all, by mice during a natural infection.

Further evidence for the lack of response of mice to the epitope recognized by the neutralizing MAbs is supplied by the data in Table 2. The results show that MAbs 159-12 and 159-18 effectively neutralized LDV present in the plasma of persistently infected mice, despite the observation that during the persistent phase of infection essentially all LDV was complexed with antibodies because of the presence of an excess of anti-VP-3 IgG (6, 30). The LDV infectivity in the plasma of infected mice was neutralized whether or not the plasma contained endogenous neutralizing activity. For example, the plasma from 55- and 107-day infected mice (Table 2) neutralized 1 $\log_{10}$ to 2 $\log_{10}$ exogenously added LDV (harvested from 1-day infected mice and thus not complexed with anti-LDV IgG), whereas plasma taken before 30 days p.i. was devoid of neutralizing activity (4). In addition, the results suggest that poor neutralization of LDV in vivo is not due to blocking of the epitope recognized by the neutralizing MAbs by antibodies or the presence of blocking antigens. Significant levels of LDV proteins are present in the circulation of LDV-infected mice, presumably being released from macrophages as a result of cytocidal replication of LDV, but these seem also to be mostly associated with anti-LDV IgG in small immune complexes (5).

In contrast to the lack of competition with the neutralizing MAbs in binding to LDV, polyclonal anti-LDV from persistently infected mice (IMP) competed with the binding of nonneutralizing MAb 159-3, although the polyclonal IMP inhibited the binding of MAb 159-3–HRP only about one-half

TABLE 2. Neutralization of LDV obtained from BALB/c mice at various times p.i. by MAbs and IRP

Anti-LDV antibody	ID_{50} after incubation with anti-LDV of LDV harvested at the following time p.i. (days)[a]:								
	1	4	20	26	29	33	40	55	107
None	$10^{9.5}$	$10^{6.5}$	$10^{6.5}$	$10^{7.0}$	$10^{7.0}$	$10^{5.0}$	$10^{6.0}$	$10^{5.5}$	$10^{6.0}$
159-12	ND[b]	$10^{5.0}$	$10^{4.5}$	$10^{5.0}$	$10^{6.0}$	$10^{3.0}$	$10^{5.5}$	$10^{4.0}$	$10^{5.5}$
159-18	$10^{5.5}$	$10^{4.0}$	$10^{3.0}$	$10^{3.0}$	$10^{3.0}$	$10^{3.0}$	$10^{4.0}$	$10^{3.0}$	$10^{4.0}$
IRP	$10^{6.0}$	$10^{4.0}$	$10^{4.0}$	$10^{5.0}$	$10^{4.0}$	$10^{3.0}$	$10^{4.0}$	ND	$10^{4.0}$

[a] Plasma was collected from a group of BALB/c mice at the indicated times p.i. with LDV_{PLA}. Samples of 1:10 dilutions thereof were incubated with MAb 159-12 or 159-18 (undiluted ascites fluid), normal mouse plasma (none), or at a 1:10 dilution of IRP and then assayed for infectious virus by endpoint titration in mice as described in Materials and Methods. All values are presented as ID_{50} per milliliter of plasma.

[b] ND, Not determined.

TABLE 3. Generation of neutralization escape variants of LDV_{PLA}

Neutralizing anti-LDV antibody	ID_{50} after incubation with anti-LDV of LDV harvested after passage no.[a]:				
	1	2	3	4	5
None	$10^{8.0}$	$10^{8.0}$	$10^{10.0}$	$10^{9.0}$	$10^{10.0}$
159-12	$10^{6.5}$	$10^{7.0}$	$10^{9.5}$	$10^{9.0}$	$10^{9.0}$
159-18	$10^{5.5}$	$10^{6.5}$	$10^{7.5}$	$10^{9.0}$	$10^{10.0}$
IRP	$10^{6.0}$	ND[b]	$10^{6.5}$	$10^{6.0}$	$10^{7.0}$

[a] LDV_{PLA} treated with MAb 159-18 was passaged in mice as described in Materials and Methods and the text. After each passage and amplification, samples of a 1:100 dilution of plasma were incubated with MAbs 159-12 and 159-18 (undiluted ascites fluid), normal mouse plasma (none), or a 1:10 dilution of IRP and then assayed for infectious LDV by endpoint titration in mice. All values are expressed as ID_{50} per milliliter of plasma. Differences in titer of 0.5 $\log_{10}$ ID_{50} are not considered significant.

[b] ND, Not determined.

as efficiently as did unlabeled MAbs 159-3, 159-5, and 159-14 (data not shown; Fig. 4).

Epitope specificity of polyclonal anti-LDV IRP. Polyclonal rabbit anti-LDV, which has been elicited by numerous injections of infectious LDV in the absence of LDV replication in rabbits, efficiently neutralizes LDV infectivity (Fig. 1; reference 7). However, IRP did not inhibit the binding of neutralizing MAb 159-12–HRP to LDV in competition experiments (data not shown), even at a concentration sufficient to neutralize 3 $\log_{10}$ LDV infectivity (diluted 1:10; Fig. 1). In contrast, polyclonal IRP was capable of inhibiting the binding of nonneutralizing MAb 159-3–HRP in a fashion similar to that of polyclonal IMP (data not shown). Polyclonal IRP was able to neutralize LDV infectivity present in the plasma of persistently infected mice as effectively as the neutralizing MAbs (Table 2). In the case of the IRP, however, we cannot distinguish whether the neutralization of infectivity was due to neutralization of LDV by anti-LDV antibodies or sensitized, infectious LDV-IgG complexes (4, 6, 30) by anti-mouse IgG antibodies present in the IRP (4). Nevertheless, the combined results of these analyses indicate that neutralization by the MAbs may involve a different epitope(s) than those recognized by polyclonal IMP generated in persistently infected mice or those epitopes recognized by polyclonal IRP.

Isolation of a neutralization escape variant. One means by which mapping of residues participating in the formation of a neutralizing epitope has been accomplished is by isolation of neutralization escape variants by serial passage of the virus in the presence of neutralizing MAbs in cell culture (10, 11, 14, 27, 35). Since no LDV-permissive cell line exists, we combined a novel in vitro and in vivo approach to isolate neutralization escape variants of LDV. LDV_{PLA} was interacted with MAb 159-18 in a standard neutralization assay, and residual infectious LDV was titrated in mice. The virus for the next round of selection was obtained at 5 days p.i. from mice inoculated with the highest dilution of LDV that caused infection. This virus was amplified by a single 1-day passage through a Swiss mouse. The amplified virus, which had been biologically cloned, was used as an inoculum for the next round of MAb selection. At each passage, the selected virus was also examined for sensitivity to neutralization by MAb 159-12 and IRP. The virus selected from LDV_{PLA} after passage 4 with MAb 159-18 was resistant to neutralization by both MAbs 159-18 and 159-12 but exhibited normal sensitivity to inhibition by IRP (Table 3). Other experiments have shown that the neutralization escape variant of LDV_{PLA} was also still neutralized by plasma from 4-month LDV-infected mice (IMP; data not shown). These results further support the conclusion that IMP and IRP recognize different neutralizing epitopes than do the MAbs.

Passive immunization with neutralizing MAbs. We previously demonstrated that passive immunization of mice with ether-extracted plasma from infected mice with neutralizing activity or MAbs to glutaraldehyde-inactivated LDV (4) had no effect on the infection of mice with LDV. The same was true for the neutralizing MAbs; they failed to protect the mice, even from the relatively low challenge dose of 100 ID_{50}s (Table 4), whether the mice were injected with anti-LDV MAb intravenously (i.v.) or intraperitoneally (i.p.) and whether LDV was injected i.v., i.p., or subcutaneously. The time courses of LDV viremia and maximum LDV titers in passively immunized and untreated mice were indistinguishable. Maximum LDV titers in plasma were observed at 1 to 2 days p.i., regardless of the route of LDV infection, and then progressively decreased over the next 2 weeks to a level about 3 $\log_{10}$ lower, which is known to persist lifelong (34). The polyclonal rabbit anti-LDV also did not protect mice from LDV infection (Table 4). In this and another experiment, the IRP seemed to delay slightly the attainment of maximum viremia when the challenge LDV was injected i.p. but had no effect when LDV was injected i.v.

TABLE 4. Lack of effect of passive immunization with neutralizing anti-LDV antibodies on infection of mice by LDV[a]

Anti-LDV antibody	Route	LDV_{PLA} challenge route	LDV (ID_{50}/ml of plasma) on the following day p.i.:			
			1	2	7	14
None		i.p.; i.v.; s.c.	$10^{9.0}$–$10^{10.0}$	$10^{9.0}$–$10^{10.0}$	$10^{6.5}$–$10^{8.5}$	$10^{7.0}$
MAb 159-18	i.v.	i.p.	$10^{10.0}$	$10^{9.5}$	$10^{8.0}$	$10^{6.5}$
	i.v.	i.v.	$10^{10.0}$	$10^{10.0}$	$10^{8.0}$	ND[b]
	i.v.	s.c.	$10^{10.5}$	$10^{10.0}$	$10^{7.0}$	ND
	i.p.	i.p.	$10^{10.0}$	$10^{10.0}$	$10^{7.5}$	ND
Rabbit polyclonal antibody	i.v.	i.p.	$10^{8.0}$	$10^{9.0}$	$10^{7.5}$	$10^{7.5}$
	i.v.	i.v.	$10^{10.0}$	$10^{10.0}$	$10^{7.0}$	ND

[a] As indicated, groups of two 8-week-old BALB/c mice were injected i.v. or i.p. with 100 μg of purified anti-LDV MAb 159-18 or 0.5 ml of a 1:10 dilution of rabbit anti-LDV. At 2 h or 1 day later, these mice and groups of control mice were injected i.p., i.v., or subcutaneously (s.c.) as indicated, with 100 ID_{50}s of LDV_{PLA} and bled at the indicated times p.i., and their pooled plasma was assayed for infectious LDV.

[b] ND, Not determined.

DISCUSSION

Fusion of spleen cells from mice primed with Formalin-inactivated LDV allowed isolation of hybridomas that secrete MAbs specific for the envelope glycoprotein VP-3 which revealed the presence of at least three distinct epitopes on this protein. One of these epitopes, which is recognized by 50% of the MAbs from this fusion, is capable of eliciting MAbs which efficiently neutralize LDV infectivity in a complement-independent fashion.

Our studies on the epitope specificity of the neutralizing MAbs, compared with polyclonal neutralizing antisera obtained from rabbits (IRP) and mice (IMP), revealed differences between the neutralizing antibody systems which may reflect recognition of different viral epitopes. Neutralization by IRP occurs with single-hit kinetics at both 4 and 37°C (7). In contrast, both IMP and the neutralizing MAbs function poorly at 4°C. This disparity could be due to differences in the mechanisms of neutralization rather than recognition of different epitopes. However, the inability of IRP to inhibit binding of the neutralizing MAbs to LDV indicates that neutralization in these two systems occurs by recognition of different epitopes.

In a similar fashion, IMP, which exhibits neutralizing activity, failed to inhibit binding of the neutralizing MAbs to LDV in competition experiments. Thus, neutralization by IMP also involves recognition of an epitope(s) different from that recognized by the neutralizing MAbs. On the other hand, both IRP and IMP are capable of inhibiting the binding of one of the nonneutralizing MAbs analyzed in this study. Thus, the epitope recognized by this nonneutralizing MAb is immunogenic in mice during a natural infection.

The conclusion that neutralizing antibodies in IRP and IMP recognize an epitope(s) different from that of the neutralizing MAbs was further supported by experiments demonstrating that a neutralization escape variant could be generated by repeated interaction with one of the neutralizing MAbs. This variant retained its sensitivity to neutralization by IRP and IMP, indicating recognition of a different epitope by the polyclonal antisera. Furthermore, the neutralizing MAbs neutralize LDV obtained from persistently infected mice at various times p.i. (including time points at which neutralizing activity is present in the plasma), which is practically all complexed with anti-LDV antibodies formed during the natural infection. Since the IRP also neutralized the infectivity of these LDV-antibody complexes present in persistently infected mice, its neutralizing antibodies seem to recognize an epitope(s) different from that of neutralizing antibodies in IMP. Thus, although these studies did not address the question of the mechanism(s) of neutralization of LDV, it appears that three distinct epitopes can function to elicit antibodies capable of neutralizing LDV.

It is apparent from the competition experiments that the epitope of Formalin-inactivated LDV that induced the neutralizing MAbs is only poorly recognized, if at all, during a natural infection. This lack of recognition could reflect differences in the immune response to LDV during a natural infection and during immunization with Formalin-inactivated LDV. Clearly, differences under the two conditions exist. For example, an active LDV infection causes an initial transient inhibition of the cellular immune response and enhancement of the humoral response to various antigens (29, 34) while also inducing a long-term general polyclonal activation of B cells (4, 6, 9). These effects are not observed during immunization with inactivated LDV (4). These alterations in immune function are not understood on a mechanistic basis but could conceivably influence the antibody response of mice to LDV both qualitatively and quantitatively. Many extrinsic and intrinsic factors are known to interact at both the T- and B-cell levels in determining the antibody repertoire that is expressed in response to a specific antigen (1, 2). Since macrophages are the primary host cells for LDV replication and also play an important role in antigen presentation, it is possible, for example, that different T-cell populations are activated during an active infection as opposed to immunization with Formalin-inactivated LDV. The concept of alteration of immune functions at the level of the antibody repertoire as a result of infection with a virus, particularly a virus such as the human immunodeficiency virus, that targets the immune system deserves further exploration in light of the current strategies in vaccine development (20).

Equally of interest in this respect is the alternate possibility that the neutralizing epitope of LDV VP-3 recognized by the MAbs is inherently nonimmunogenic in mice but is rendered immunogenic by Formalin treatment, perhaps by a slight conformational change or denaturation. Consistent with this view are the findings that no antibody to this epitope is made in rabbits or mice during immunization with glutaraldehyde-inactivated LDV. However, in these cases one could argue that the lack of response reflects a species difference (1, 2) or epitope inactivation by glutaraldehyde. Glutaraldehyde is a much stronger protein cross-linking agent than Formalin (17). It is also unclear whether the apparently altered immunogenicity of this epitope by Formalin is mediated at the B- or T-cell level. Nevertheless, differences in antibody response have also been observed after immunization with live and inactivated poliovirus (33).

The neutralizing MAbs recognize LDV antigens coated onto ELISA plates in the form of intact virions which may have been denatured to various degrees (15, 19, 26). Thus, the positive ELISA reaction does not mandate that the neutralizing MAbs react with a native virus (20a). However, this is probably the case, since they neutralize LDV in suspension. Attempts to demonstrate that the neutralizing MAbs interact with a native virus by addition of excess suspended LDV in the competition assay (20a) have been unsuccessful; all dilutions of suspended, purified LDV tested have enhanced binding of the neutralizing MAbs, possibly because of antigen adherence to the plate during incubation.

Regardless of the nature of the neutralizing epitope that elicits the neutralizing MAbs, it is unclear whether the inability of mice to respond to it during a natural infection contributes to establishment of lifelong persistence of LDV in mice. What speaks against such a role is the finding that passive immunization with neutralizing MAbs or rabbit anti-LDV IgG fails to protect mice from LDV infection or alter the progression of the infection. Thus, although it is possible that chemical treatments of other viruses can enhance the immunogenicity of normally silent neutralizing epitopes in a susceptible host, it is not clear that the antibodies elicited by these epitopes would be protective. In the case of LDV, its resistance to antibody-dependent complement lysis mediated by either neutralizing or nonneutralizing antibodies might be more important in its resistance to host defense mechanisms than inefficient production of neutralizing antibodies per se. It is also unclear whether such chemical treatments of viruses could generate increases in cellular immune responses that might play a role in virus clearance.

ACKNOWLEDGMENTS

We thank Ron Jemmerson for technical advice, useful discussions, and helpful editorial comments and Linda Colbeth for secretarial assistance.

This work was supported by U.S. Public Health Service research grant AI15267 and training grant CA09138 (J.T.H.) from the National Institutes of Health.

LITERATURE CITED

1. **Berzofsky, J. A.** 1983. T-B reciprocity. An Ia-restriced epitope-specific circuit regulating T-cell–B-cell interaction and antibody specificity. Surv. Immunol. Res. **2**:223–229.
2. **Berzofsky, J. A.** 1985. Intrinsic and extrinsic factors in protein antigenic structure. Science **229**:932–940.
3. **Brinton-Darnell, M., and P. G. W. Plagemann.** 1975. Structure and chemical-physical characteristics of lactate dehydrogenase-elevating virus and its RNA. J. Virol. **16**:420–433.
4. **Cafruny, W. A., S. P. K. Chan, J. T. Harty, S. Yousefi, K. Kowalchyk, D. McDonald, B. Foreman, G. Budweg, and P. G. W. Plagemann.** 1986. Antibody response of mice to lactate dehydrogenase-elevating virus during infection and immunization with inactivated virus. Virus Res. **5**:357–375.
5. **Cafruny, W. A., D. Heruth, M. Jaqua, and P. G. W. Plagemann.** 1986. Immunoglobulins which bind to uncoated ELISA plate surfaces: appearance in mice during infection with lactate dehydrogenase-elevating virus and in human anti-nuclear antibody-positive sera. J. Med. Virol. **19**:175–186.
6. **Cafruny, W. A., and P. G. W. Plagemann.** 1982. The immune response to lactate dehydrogenase-elevating virus: isolation of infectious virus-immunoglobulin G complexes and quantitation of specific antiviral immunoglobulin G response in wild-type and nude mice. Infect. Immun. **37**:1001–1006.
7. **Cafruny, W. A., and P. G. W. Plagemann.** 1982. Immune response to lactate dehydrogenase-elevating virus: serologically specific rabbit neutralizing antibody to the virus. Infect. Immun. **37**:1007–1012.
8. **Cooper, N. R.** 1979. Humoral immunity to viruses. Compr. Virol. **15**:123–170.
9. **Coutelier, J. P., E. van Roost, P. Lambotte, and J. van Snick.** 1986. The murine antibody response to lactate dehydrogenase-elevating virus. J. Gen. Virol. **67**:1099–1108.
10. **Dalziel, R. G., P. W. Lampert, P. J. Talbot, and M. J. Buchmeier.** 1986. Site-specific alteration of murine hepatitis virus type 4 peplomer glycoprotein E2 results in reduced neurovirulence. J. Virol. **59**:463–471.
11. **Diamond, D. C., B. A. Jameson, J. Bonin, M. Kohara, S. Abe, H. Itoh, T. Komatsu, M. Arita, S. Kuge, A. Nomoto, A. D. M. E. Osterhaus, R. Crainic, and E. Wimmer.** 1985. Antigenic variation and resistance to neutralization in poliovirus type 1. Science **229**:1090–1093.
12. **Engvall, E.** 1980. Enzyme immunoassay ELISA and EMIT. Methods Enzymol. **70**:419–439.
13. **Ey, P. L., S. J. Prowse, and C. R. Jenkin.** 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry **15**:429–436.
14. **Fleming, J. O., M. D. Trousdale, F. A. K. El-Zaatari, S. A. Stohlman, and L. P. Weiner.** 1986. Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. J. Virol. **58**:869–875.
15. **Friguet, B., L. Djavadi-Ohaniance, and M. E. Goldberg.** 1984. Some monoclonal antibodies raised with a native protein bind preferentially to the denatured antigen. Mol. Immunol. **21**:673–677.
16. **Gollins, S. W., and J. S. Porterfield.** 1986. A new mechanism for the neutralization of enveloped viruses by antiviral antibody. Nature (London) **321**:244–246.
17. **Haase, A. T., M. Brahic, and L. Stowring.** 1984. Detection of viral nucleic acids by *in situ* hybridization. Methods Virol. **7**: 189–226.
18. **Harty, J. T., S. P. K. Chan, and P. G. W. Plagemann.** 1987. Characteristics of monoclonal antibodies to the lactate dehydrogenase-elevating virus. Intervirology **27**:53–60.
19. **Hollander, Z., and E. Katchalski-Katzir.** 1986. Use of monoclonal antibodies to detect conformational alterations in lactate-dehydrogenase isoenzyme 5 on heat denaturation and on adsorption to polystyrene plates. Mol. Immunol. **23**:927–933.
20. **Hu, S.-L., P. N. Fultz, H. M. McClure, J. W. Eichberg, E. K. Thomas, T. Zarling, M. C. Singhal, S. G. Kusowski, R. B. Swenson, D. C. Anderson, and G. Todaro.** 1987. Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. Nature (London) **378**:721–724.

20a. **Jemmerson, R.** 1988. Antigenicity and native structure of globular proteins: low frequency of peptide reactive antibodies. Proc. Natl. Acad. Sci. USA **85**:9180–9184.

21. **Lachmann, P. J.** 1985. Antibody and complement in viral infections. Br. Med. Bull. **41**:3–6.
22. **Mandel, B.** 1979. Interaction of viruses with neutralizing antibodies. Compr. Virol. **15**:37–121.
23. **Martinez, D., M. A. Brinton, T. G. Tachovsky, and A. H. Phelps.** 1980. Identification of lactate dehydrogenase-elevating virus as the etiological agent of genetically restricted, age-dependent polioencephalomyelitis of mice. Infect. Immun. **27**:979–987.
24. **Massey, R. J., and G. Schochetman.** Monoclonal antibodies to viruses, p. 29–38. *In* M. H. V. van Regenmortel and A. R. Neurath (ed.), Immunochemistry of viruses. Elsevier Biomedical Press, Amsterdam.
25. **McDonald, T. L.** 1982. Isolation of Clq-binding virus-antibody immune complexes from lactic dehydrogenase virus (LDV)-infected mice. Immunology **45**:365–370.
26. **Mierendorf, R. C., Jr., and R. L. Dimond.** 1983. Functional heterogeneity of monoclonal antibodies obtained using different screening assays. Anal. Biochem. **135**:221–229.
27. **Minor, P. D., D. M. A. Evans, M. Ferguson, G. C. Schild, G. Westrop, and J. W. Almond.** 1985. Principal and subsidiary antigenic sites of VP1 involved in the neutralization of poliovirus type 3. J. Gen. Virol. **65**:1159–1165.
28. **Murphy, W. H., J. F. Nawrocki, and L. R. Pease.** 1983. Age-dependent paralytic viral infection in C58 mice: possible implications in human neurological disease. Prog. Brain Res. **59**: 291–303.
29. **Notkins, A. L.** 1971. Enzymatic and immunological alterations in mice infected with lactic dehydrogenase virus. Am. J. Pathol. **64**:733–746.
30. **Notkins, A. L., S. Mahar, C. Scheele, and J. Goffman.** 1966. Infectious virus-antibody complexes in the blood of chronically infected mice. J. Exp. Med. **124**:81–97.
31. **Oldstone, M. B. A., J. J. Buchmeyer, M. V. Doyle, and A. Tishon.** 1980. Virus-induced immune complex disease: specific antiviral antibody and Clq binding material in the circulation during persistent lymphocytic choriomeningitis virus infection. J. Immunol. **124**:831–838.
32. **Plagemann, P. G. W., K. G. Gregory, H. E. Swim, and K. K. W. Chan.** 1963. Plasma lactic dehydrogenase-elevating agent of mice: distribution in tissues and effect on lactic dehydrogenase isoenzyme patterns. Can. J. Microbiol. **9**:75–86.
33. **Roivainen, M., and T. Hovi.** 1987. Intestinal trypsin can significantly modify antigenic properties of polioviruses: implications for the use of inactivated poliovirus vaccine. J. Virol. **61**:3749–3753.
34. **Rowson, K. E. K., and B. W. J. Mahy.** 1985. Lactate dehydrogenase-elevating virus. J. Gen. Virol. **66**:2297–2312.
35. **Stec, D. W., A. Waddell, C. S. Schmaljohn, G. A. Cole, and A. L. Schmaljohn.** 1986. Antibody-selected variation and reversion in Sindbis virus neutralization epitopes. J. Virol. **57**:715–720.
36. **Stueckemann, J. A., M. Holth, W. J. Swart, K. Kowalchyk, M. S. Smith, A. J. Wolstenholme, W. A. Cafruny, and P. G. W. Plagemann.** 1982. Replication of lactate dehydrogenase-elevating virus in macrophages. 2. Mechanism of persistent infection in mice and cell culture. J. Gen. Virol. **59**:263–272.
37. **Stueckemann, J. A., D. M. Ritzi, M. Holth, M. S. Smith, W. J. Swart, W. A. Cafruny, and P. G. W. Plagemann.** 1982. Replication of lactate dehydrogenase-elevating virus in macrophages. 1. Evidence for cytocidal replication. J. Gen. Virol. **59**:245–262.
38. **Thomas, A. A. M., R. Vrijsen, and A. Boeyé.** 1986. Relationship between poliovirus neutralization and aggregation. J. Virol. **59**: 479–485.