Mode of neutralization of lactate dehydrogenase-elevating virus by polyclonal and monoclonal antibodies

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Summary. Neutralization of the infectivity of [^{3}H]uridine-labeled lactate dehydrogenase-elevating virus (LDV) by polyclonal mouse or rabbit antibodies to the envelope glycoprotein of LDV, VP-3, or by neutralizing monoclonal antibodies (mAb) that recognize a different epitope on VP-3 than the polyclonal antibodies correlated with an increase in the sedimentation rate of LDV from 230 S to $\geqslant$ 270 S. Incubation of LDV with normal mouse plasma or non-neutralizing mAbs to LDV VP-3 had no effect on its sedimentation rate. Similarly, incubation of a neutralization escape variant of LDV with the mAb used in its selection had no effect on its sedimentation rate, whereas neutralization of this variant by polyclonal mouse or rabbit anti-VP 3 antibodies increased the sedimentation rate. Neutralization of LDV infectivity was only observed at high antibody/virion ratios and often was followed by loss of the viral RNA. The results suggest that neutralization of LDV infectivity results from binding of multiple antibody molecules that recognize specific epitopes on the viral envelope glycoprotein and ultimately leads to disintegration of the virions.

Introduction

The infection of animal cells by a virus is generally mediated by specific surface protein(s) of the virus and interaction of antibodies with certain antigenic sites on this protein may lead to abolishment or neutralization of viral infectivity. However, the mechanism of neutralization of viral infectivity is still poorly understood at the molecular level and may differ for different viruses and different antibodies [7, 17, 20–22, 27, 39]. In the case of non-enveloped viruses, the general concept has been that binding of one or a few antibody molecules causes a conformational change in the capsid or cross-linking of the capsomers that renders the virus non-infectious, but recent work suggests that in many cases virus aggregation may also play an important role in neutralization [5,

17, 23, 35, 39]. Less clear is the mechanism of antibody neutralization of enveloped viruses. Neutralizing antibody binding to virions may prevent interaction with host cell receptors, but more often aborts infection at the level of penetration – uncoating of the virions [7, 8, 10, 27]. In the case of influenza virus, neutralizing antibodies bind to epitopes adjacent to the receptor binding (virus attachment) site and the mode of in vitro neutralization depends on the antibody/virion ratio [27]. At low antibody/virion ratios neutralization of infectivity is associated with virion aggregation and inhibition of virion attachment to cell receptors, whereas at high antibody/virion ratios neutralized virions remain monodisperse and infection is blocked at a step following internalization by the cells [27]. Furthermore, in some instances neutralization of enveloped viruses may be due to antibody-dependent complement lysis [7, 20, 21].

The finding that the neutralization of many viruses by antibodies followed apparent single hit kinetics had originally suggested that the binding of a single antibody molecule can neutralize the infectivity of a virion [21], but most recent studies have raised questions as to the accuracy of such dose-response curves and suggest that effective neutralization of a virion requires more than one antibody molecule [17, 23].

The present study inquires into the mechanism of neutralization of lactate dehydrogenase-elevating virus (LDV) by polyclonal and monoclonal antibodies to the single envelope glycoprotein of LDV, VP-3 [3, 4, 12]. LDV has been tentatively classified as a togavirus [38], but recent studies suggest that it has a gene organization similar to that of equine arteritis virus and thus of coronaviruses [9, 19, 29]. It establishes an asymptomatic infection in mice that invariably lasts for the life of the animal [6, 29, 31, 32]. LDV replication in vitro and most likely also in vivo is restricted to a subpopulation of tissue macrophages [29, 33, 34, 36] possessing a surface protein that acts as LDV receptor [2, 16, 18, 26, 29]. Though LDV replication in macrophages is rapidly cytocidal, a persistent infection in mice can be maintained by the generation of new permissive macrophages throughout the animal's life [25, 29, 33, 34].

How LDV escapes host immune responses is still unclear. Mice generate a rapid and strong antibody response to several epitopes on VP-3, but antibodies that neutralize LDV in vitro appear in the circulation of mice only 1–2 months postinfection (p.i.) and seem relatively inefficient in LDV neutralization [3, 25, 29, 32]. VP-3 varies in size from 25–40 kDa, probably because of different degrees of glycosylation [3, 11, 12, 29]. It is quite small compared to the envelope glycoproteins of other viruses, which may play a role in the mechanism by which LDV escapes host immune functions. In attemps to elucidate these mechanisms we have generated batteries of mouse monoclonal antibodies (mAb) to LDV VP-3 [11, 12]. Five mAbs generated to formalin-inactivated LDV have been found to interact with a single antigenic site on VP-3 of intact virions and to neutralize their infectivity, but the epitope recognized by the neutralizing mAbs is non-immunogenic during a natural infection [12]. Two additional epitopes on VP-3 are recognized by non-neutralizing mAbs to formalin-inac-

tivated LDV. Neither polyclonal nor monoclonal neutralizing anti-LDV antibodies protect mice from LDV infection when passively transferred by any route, even at very high concentrations [3, 12, 29]. The present study shows that the in vitro neutralization of LDV infectivity for mice correlates with a marked increase in the sedimentation rate of the virions, rather than the formation of large aggregates. It seems to involve the binding of multiple antibody molecules per virion and most likely cross-linking of viral proteins and structural alterations that ultimately lead to the disintegration of the virions.

Materials and methods

Mice

Female Swiss mice (4–6 weeks of age) were obtained from BioLabs, Inc., St. Paul, MN. BALB/c mice were bred in the animal facility of the Department of Microbiology, University of Minnesota.

Viruses

Groups of 50 to 150 Swiss mice were infected with the strain of LDV originally isolated in this laboratory (LDV$_P$; [1]). Their plasma was harvested 1 day postinfection (p.i.) and used as inoculum for macrophage cultures. The isolation of a variant of LDV$_P$ that is resistant to neutralization by neutralizing anti-LDV mAbs (LDV$_{P\text{-}NE}$) has been reported previously [12]. LDV$_{P\text{-}NE}$ was propagated in Swiss mice as described for wild type virus. LDV concentrations were determined by an end point dilution assay in mice as described previously [28].

[^{3}H]uridine-labeled LDV was prepared as described previously [1, 36]. In brief, one-day primary cultures of peritoneal macrophages from Swiss or BALB/c mice were infected with about 100 50% infectious doses (ID$_{50}$) of LDV/cell. About 3 h p.i. the culture fluid was supplemented with 10 µCi of [5-^{3}H]uridine ($\sim$ 30 Ci/mmol; Moravek Biochemicals, Brea, CA)/ml and then harvested 18 h p.i. and clarified by low speed centrifugation. The fluid was stored at $-70\,°C$ until further analyzed.

Semliki Forest virus (SFV; kindly supplied by Dr. S. I. T. Kennedy) was propagated in a line of baby hamster kidney cells (BHK) [37]. [^{3}H]uridine-labeled SFV was prepared as described for LDV, except that the virus was propagated in BHK cells.

Anti-LDV quantitation

Anti-LDV antibodies were quantitated by a fluorescent antibody (FA) staining assay as described previously [3]. Peritoneal macrophages from 4–6 week old BALB/c mice were cultured on coverslips for 1 day, then infected with 100–1,000 ID$_{50}$ of LDV$_P$/cell and fixed at 8 h p.i. in acetone for 10 min. The coverslips were sequentially incubated with two-fold dilutions of plasma from infected mice and a 1:50 dilution of fluorescein isothiocyanate-conjugated (FITC) rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA) and examined in a fluorescence microscope. The FA titer was expressed as the reciprocal of the highest antibody dilution that yielded recognizable staining of 5–15% of the total macrophages in LDV-infected cultures without staining any cells in uninfected cultures. Only FA titers > 32 are significant.

Anti-LDV antibodies

Neutralizing mouse and rabbit anti-LDV polyclonal antibodies consisted of plasma from five-month LDV-infected mice (IMP) and from rabbits immunized with LDV (IRP), re-

spectively [3, 4]. Anti-LDV neutralizing mAbs (159-7, -12, -16, -18, -19) and non-neutralizing mAbs (159-3, -4, -5, -13, -14) have been generated to formalin-inactivated LDV_P in a previous study [12]. The FA titers of IMP and the ascites fluid containing the various mAbs were about 4,000 and $\geqslant$ 15,000, respectively [3, 12, 25].

Reaction of ^{3}H-labeled LDV with anti-LDV antibodies and analysis by zone sedimentation in sucrose density gradients

Samples of 0.5 ml of suspensions of [^{3}H]uridine-labeled LDV ($\sim 10^{8.5}$ ID_{50}/ml) were mixed with 0.1 or 0.2 ml of normal mouse plasma or of various anti-LDV antibody preparations and additional components as indicated in appropriate experiments. If not indicated otherwise, the mixtures were incubated at 37 °C for 4 h and at 4 °C for 1 h. Then a 10 µl sample was removed from each mixture for titration of residual LDV infectivity by mouse inoculation [3] to assess the degree of virion neutralization. The remainder of each mixture was layered onto a 0.15–0.9 M gradient of sucrose in TNE (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.5) over 0.5 ml of a 2 M sucrose cushion. The gradients were centrifuged in a SW 41 rotor in a Beckman ultracentrifuge at 36,000 rpm for 1.5 or 2 h at 4 °C. Fractions of 0.35 ml were collected from the gradients and analyzed for radioactivity in acid-insoluble material [36].

Results

Figure 1 A illustrates that incubation of [^{3}H]uridine-labeled LDV at 37 °C for 4 h with anti-LDV neutralizing mAbs 159-18 and 159-7 (left frame), with plasma from five-month LDV-infected mice (IMP), or plasma from a rabbit immunized with LDV (IRP; right frame) increased the rate of its sedimentation 20–25%. This increase in sedimentation rate correlated with neutralization of LDV infectivity as measured by mouse inoculations (see values in Fig. 1). A similar incubation of ^{3}H-labeled LDV with normal mouse plasma (NMP) or the non-neutralizing mAb 159-3 had no effect on the sedimentation of LDV (Fig. 1 A), even though the latter also binds to VP-3 and the FA staining titer of the 159-3 mAb ascites fluid was as high as that of the ascites fluid containing the neutralizing mAbs ($\geqslant$ 15,000). Neutralizing mAbs 159-7 and 159-18 react with the same antigenic site on LDV VP-3 and are of IgG 2a isotype [12]. However, neutralization of LDV by incubation with two other mAbs directed to this site but of isotype IgG 2b (159-12) or IgG 1 (159-19) was also associated with an increase in virion sedimentation rate, whereas incubation with additional non-neutralizing mAbs to VP-3 failed to elicit this effect (data not shown). As pointed out already, the IMP and IRP do not contain significant levels of antibodies to the antigenic site recognized by the neutralizing mAbs [12]; thus neutralization of LDV by antibodies to at least two epitopes on VP-3 correlated with an increased sedimentation rate. From the sedimentation profiles in Fig. 1 A, we estimated that neutralization was associated with an increase in sedimentation coefficient of LDV from about 230 S to 270–280 S. The sedimentation coefficient of 230 S for LDV was estimated from a comparison of its sedimentation rate to that of SFV (264 S; reported in [14, 15]; see Fig. 1 B). Only little radiolabeled LDV was recovered in the pellets of the sucrose gradients, which
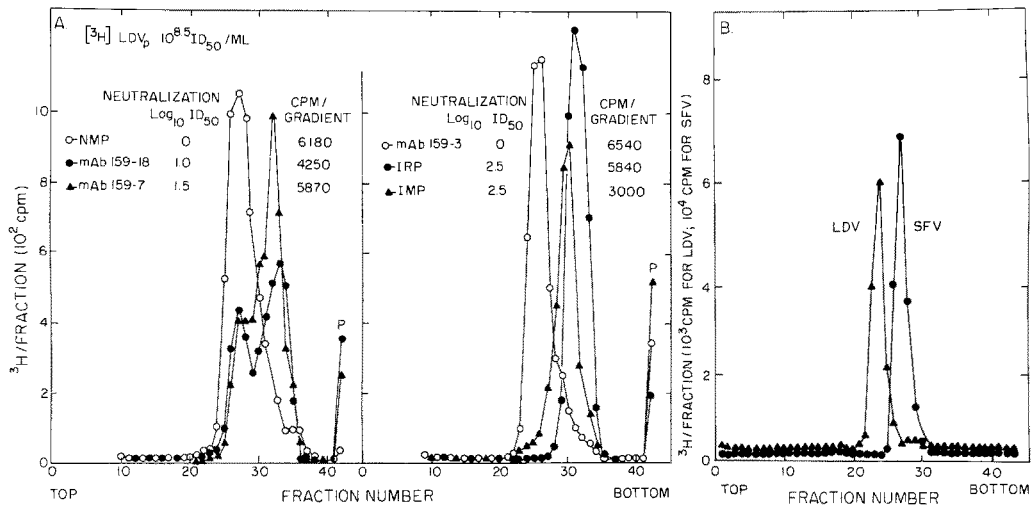
Fig. 1. Effect of incubation of LDV with anti-LDV antibodies on virus infectivity and sedimentation rate (**A**) and comparison of LDV and SFV by zone sedimentation (**B**). **A** Samples of 0.5 ml of a suspension of [^{3}H]uridine-labeled LDV ($10^{8.5}$ ID$_{50}$/ml) were mixed with 0.1 ml of normal mouse plasma (NMP), IMP or IRP, or 0.2 ml of ascites fluid containing mAbs 159-3, -7 or -18. The mixtures were incubated sequentially at 37 °C for 4 h and at 4 °C for 1 h. Then a 10 µl sample was removed and assayed for infectious LDV and the remainder was layered onto a 0.15–0.9 M gradient of sucrose in TNE. The gradients were centrifuged in a SW 41 rotor at 36,000 rpm (4 °C) for 2 h and fractions from the gradient and the pellet (*P*) were analyzed for radioactivity in acid-insoluble material. The total radioactivity recovered from the gradients is stated for each. Neutralization of LDV is defined as the reduction in LDV infectivity as a result of incubation with anti-LDV antibodies (in log$_{10}$ ID$_{50}$). **B** [^{3}H]uridine labeled LDV and SFV were analyzed by zone sedimentation in sucrose density gradients as described above

are expected to contain aggregated virions, and antibody neutralization did not significantly increase the amount of LDV recovered in the pellets (Fig. 1 A).

In some sedimentation analyses we observed the formation of an additional even more rapidly sedimenting fraction in neutralized LDV preparations (Fig. 2 A) and, in addition, prolonged incubation of [^{3}H]uridine-labeled LDV with neutralizing antibodies generally resulted in a considerable, but variable, loss of total radioactivity (see Figs. 1 A and 2 A). Both the increase in sedimentation rate and loss of radioactivity during neutralization developed only slowly during neutralization of LDV by neutralizing antibodies (Fig. 2 A). The loss of radioactivity seems to reflect a modification in virion structure that renders the viral RNA susceptible to nuclease degradation, since the radioactivity lost from virions was not recovered in other fractions of the sucrose density gradient (Fig. 2 A, and data not shown). Complement probably did not play a role in the degradation of neutralized virions since the loss of radioactivity during incubation with IMP was observed whether or not the IMP had been preheated (56 °C, 30 min) and since addition of rabbit complement to the LDV-neutralizing antibody incubation mixture had no significant effect on the change
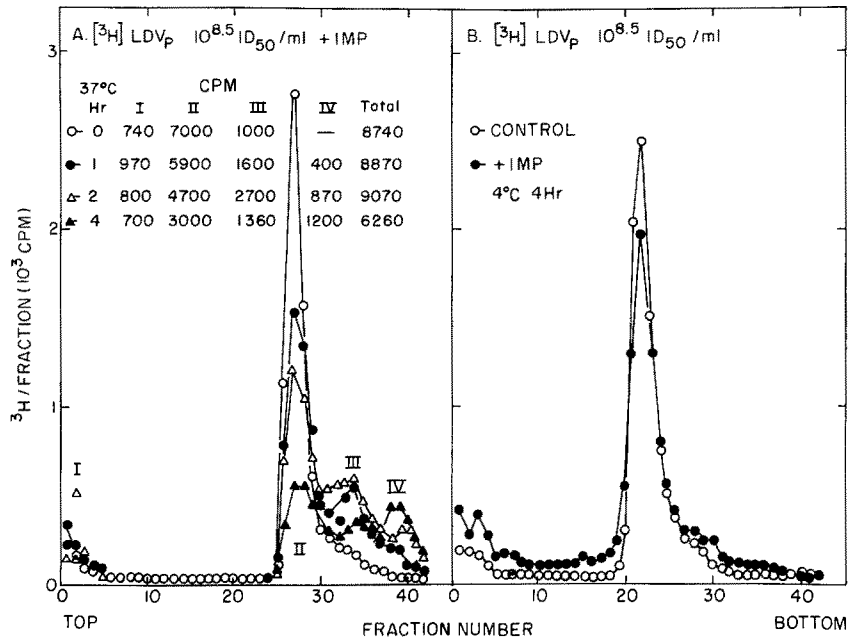
Fig. 2. Change in sedimentation rate of LDV as a function of incubation with IMP at 37 °C (**A**) and lack of effect of incubation with IMP at 4 °C (**B**). Samples of a suspension of [^{3}H]-uridine-labeled LDV ($10^{8.5}$ ID_{50}/ml) were mixed with 0.1 ml of NMP or IMP. The mixtures were incubated for the indicated time periods at 37 °C (**A**) or 4 °C (**B**) and then layered onto gradients of 0.15–0.9 M sucrose over a 2 M sucrose cushion. The gradients were centrifuged in a SW 41 rotor at 36,000 rpm (4 °C) for 1.5 h and fractions from the gradient were analyzed for radioactivity in acid-insoluble material. The values stated in **A** refer to the total radioactivity recovered in pooled fractions I, II, III, and IV of each gradient

in virion sedimentation rate or loss of radioactivity (data not shown). Previously it has been demonstrated that LDV-antibody complexes fix complement only poorly [24] and that complement has no significant effect on the neutralization of LDV by anti-LDV polyclonal or monoclonal antibodies [3, 12].

The presence of 2.5 mg RNAse A/ml in the incubation mixture had no effect on the sedimentation properties or infectivity of LDV and also did not affect the neutralization of LDV by anti-LDV monoclonal (159-12) or polyclonal (IMP) antibodies (data not shown). The results indicate that the more rapidly sedimenting particles in neutralized LDV preparations contain virions in which the viral RNA is still protected from RNAse attack. Thus they cannot represent aggregated nucleocapsids since the viral RNA in the latter is sensitive to nuclease degradation [1].

The following other evidence causally links the increase in sedimentation rate of LDV with neutralization of its infectivity. First, incubation of LDV with IMP at 4 °C, which is known to be ineffective in neutralization [4, 12], failed to result in a shift in sedimentation rate (Fig. 2 B). Second, incubation of a variant of LDV, which is resistant to neutralization by the neutralizing mAbs (neutralization escape variant, LDV$_{P-NE}$; [12]) with neutralizing mAb 159-7,

like incubation with the non-neutralizing mAb 159-3, had no effect on its sedimentation rate or its infectivity (Fig. 3). VP-3 of LDV$_{P-NE}$ does not seem to react with the neutralizing mAbs. As shown in Fig. 4, although permissive macrophages infected with LDV$_{P-NE}$ (5–10% of the total macrophages in these cultures), reacted with IMP and the non-neutralizing anti-VP-3 mAb 159-3 (Fig. 4 B and D), they failed to react with the neutralizing mAb 159-12 (Fig. 4 F). In contrast, permissive macrophages infected with wild type LDV$_P$ reacted equally with all three anti-LDV antibodies (Fig. 4 A, C, and E). LDV$_{P-NE}$ is still effectively neutralized by IMP and IRP [12] and its neutralization by IRP was associated with an increase in sedimentation rate (Fig. 3).

Third, neutralization of LDV by polyclonal or monoclonal antibodies and the induced change in sedimentation rate both require high antibody concentrations (undiluted ascites fluid or IMP). We have shown previously [4, 12] that diluting these antibody preparations 1:10 reduces neutralizing activity ≥ 90% and little or no neutralization is observed at a dilution of 1:100, whereas the FA titer of the mAb ascites fluids and IMP are ≥ 15,000 and 4,000 respectively. Similarly, little change in sedimentation rate was observed upon incubation of LDV with a 1:10 dilution of IMP (data not shown).

Monovalent Fab fragments of neutralizing mouse antiviral antibodies have been found to exhibit greatly reduced neutralization of rhinoviruses [5] or not to neutralize alphaviruses [30]. Neutralizing activity was largely restored by incubation of virus-Fab mixtures with anti-mouse IgG. One explanation of the latter finding is that cross-linking of viral surface proteins plays a role in virus neutralization. We have been unable to investigate whether Fab fragments of
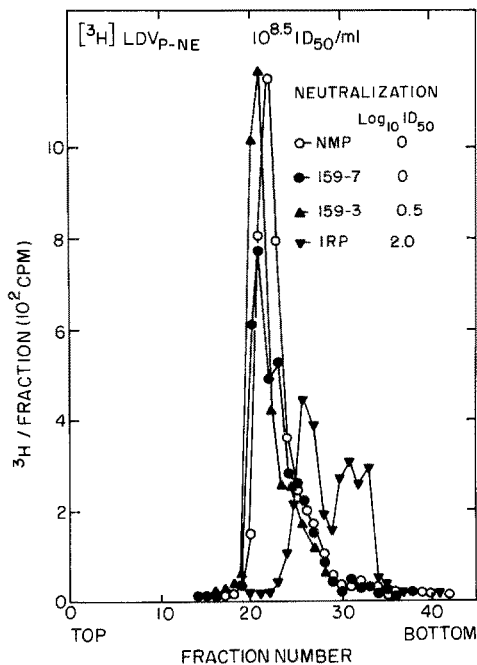

Fig. 3. Effect of incubation with anti-LDV antibodies on the sedimentation rate of neutralization escape variant LDV$_{P-NE}$. Samples of 0.5 ml of a suspension of [^{3}H]uridine-labeled LDV$_{P-NE}$ ($10^{8.5}$ ID$_{50}$/ml) were mixed with 0.1 ml of NMP or IRP, or 0.2 ml of ascites fluid containing mAbs 159-3 or -7. The mixtures were sequentially incubated at 37 °C for 4 h and 4 °C for 1 h and then analyzed for LDV infectivity and sedimentation rate as described in the legend to Fig. 1, except that the gradients were only centrifuged for 1.5 h
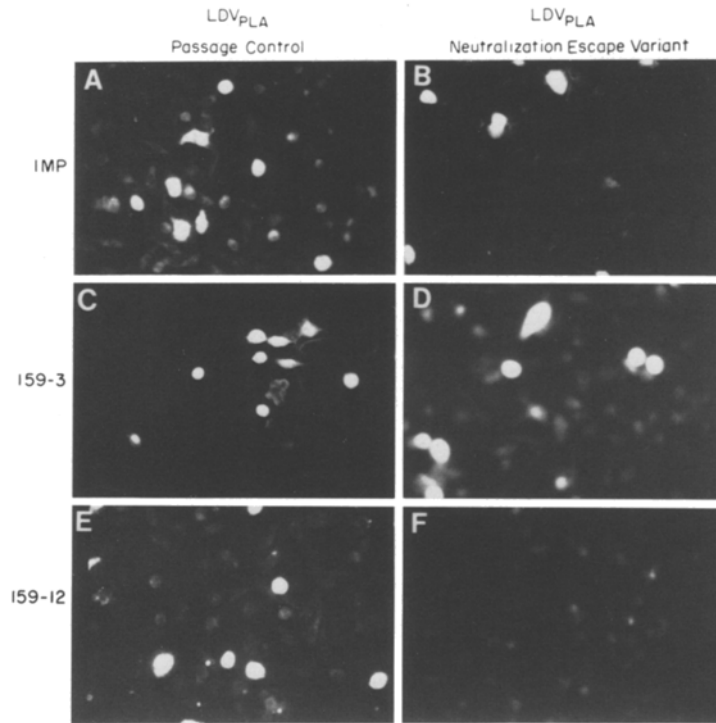
Fig. 4. FA staining of macrophage cultures infected with LDV$_P$ (**A, C, E**) or LDV$_{P-NE}$ (**B, D, F**). Infected macrophage coverslip cultures were acetone-fixed 8 h p.i. and then incubated sequentially with a 1 : 100 dilution of IMP (**A, B**), non-neutralizing mAb 159-3 (**C, D**) or neutralizing mAb 159-12 (**E, F**), and FITC anti-mouse IgG as described under Materials and methods

our neutralizing mAbs neutralize LDV infectivity, because upon their purification by protein G chromatography [25], the mAbs, regardless of isotype, completely lost their in vitro neutralizing activity, while retaining their activity to interact with LDV-infected macrophages in the FA staining assay or with LDV in an ELISA (data not shown) and to protect anterior horn motor neurons from LDV infection in C 58 mice [13]. Even at very high concentrations (1–2.5 mg/ml) purified neutralizing mAbs 159-12 (IgG 2b), 159-19 (IgG 1) or 159-7 (IgG 2a) failed to neutralize LDV infectivity significantly and the neutralizing activity of mAb 159-12 was not restored by inclusion of ascites fluid from an unrelated hybridoma, normal mouse serum or rabbit or guinea pig complement in the incubation mixture (data not shown).

Discussion

Our data demonstrate a strong correlation between LDV neutralization by anti-LDV polyclonal and monoclonal antibodies to at least two epitopes of the envelope glycoprotein VP-3 and increased sedimentation rate of the virus. The results suggest that LDV neutralization is not a result of virion aggregation, but instead results from a binding of multiple antibodies to each virion. On the basis of the S value of the neutralized virions, we estimate that at least ten

antibodies are bound per neutralized virion. Furthermore, to achieve neutralization the virions must be incubated with high concentrations of neutralizing antibodies. For an about 90% neutralization, 10^8 ID_{50} of LDV must be incubated at 37 °C with at least 10^{14} neutralizing anti-LDV mAb molecules (0.02 ml of ascites fluid containing 2 mg anti-LDV mAb/ml). Thus, antibody binding that results in neutralization of LDV is rather inefficient. This is also indicated by the finding that neutralization by anti-LDV antibodies occurs at 37 °C but not at 4 °C [4].

Long-term incubation of LDV with neutralizing antibodies seems to lead to degradation of viral RNA, presumably as a consequence of antibody-induced alterations in virion structure and nuclease attack. Most likely it involves the sloughing off of the viral envelope, thus exposing the nucleocapsid in which the viral RNA is not protected from nuclease attack [1]. The envelope of LDV is unusually fragile; it is readily removed by treatment of LDV with non-ionic detergents at concentrations as low as 0.01% and by exposure of LDV to hypotonic solutions [1]. Degradation of viral RNA, however, is probably a secondary effect, since it seems to occur less rapidly than neutralization of infectivity and the rapidly sedimenting fractions contain intact virions in which the viral RNA is still protected from RNAse degradation. The nature of the structural alterations induced by the binding of neutralizing anti-LDV antibodies is unclear and it is not known whether the change is triggered by the binding of the antibodies per se or requires additional interactions. The non-neutralizing anti-LDV mAbs are also specific for the envelope glycoprotein VP-3, but they may not bind to intact virions because of an inaccessibility of the epitopes they recognize or their binding fails to induce structural alterations or cross-linking of VP-3 that may be required for neutralization. Whether cross-linking of VP-3 by antibodies plays a role in neutralization could not be investigated, because protein G purification of the neutralizing mAbs caused a loss of neutralizing activity. Since protein G-purified mAbs exhibit undiminished interaction with LDV in an ELISA and with LDV-infected macrophages in the FA staining the loss in neutralizing activity could indicate that an additional component(s) present in anti-LDV preparations may play a role in neutralization of LDV infectivity in vitro, which is removed during protein G purification. However, ascites fluid from unrelated hybridomas, normal mouse serum or complement failed to restore the neutralizing activity of these antibodies.

It also remains to be determined whether neutralization of LDV infectivity involves blockage of LDV interaction with its macrophage receptor(s) or of penetration/uncoating. This question is difficult to approach in the case of LDV, because LDV infections are restricted to a subpopulation of macrophages and macrophages can also be infected via Fc receptors by LDV-antibody complexes [4, 16].

Acknowledgements

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