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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

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Lactate dehydrogenase-elevating virus (LDV) causes a normally benign persistent infection of mice, resulting in a life-long viremia characterized by the presence of circulating infectious immune complexes, impaired clearance of certain enzymes from the blood, and modification of the host immune response to various heterologous antigens. In this study, we isolated infectious immunoglobulin G (IgG)-LDV complexes in the plasma of persistently infected mice by adsorption to and elution from protein A-Sepharose CL-4B. We found that practically all infectious LDV in the plasma of persistently infected mice is complexed to IgG. LDV infectivity in these complexes was partially neutralized, but could be reactivated by treatment with 2-mercaptoethanol. We also quantitated total plasma IgG and anti-LDV IgG in wild-type and nude Swiss and BALB/c mice as a function of the time after infection with LDV by radial immunodiffusion and an enzyme-linked immunosorbent assay, respectively. Total plasma IgG levels nearly doubled in BALB/c mice during 150 days of infection. IgG levels in uninfected nude mice were only 20% of those in uninfected BALB/c mice, but during infection with LDV increased to approximately those found in uninfected BALB/c mice. Anti-LDV IgG levels were almost as high in nude mice as in normal BALB/c mice. Isoelectric focusing of purified IgG from BALB/c mice showed that LDV infection resulted in the enhanced synthesis of all 16 normal IgG fractions that we could separate by this method, which suggests that LDV infection results in polyclonal activation of IgG-producing lymphocytes.

Lactate dehydrogenase-elevating virus (LDV) is a nonflavi, nonalpha mouse togavirus (3, 32), whose replication is apparently limited to a subpopulation of macrophages (2, 35, 36). The primary infection of mice results in the development of plasma virus titers of up to 10^{10} median infectious doses (ID_{50}) per ml which peak at 16 to 24 h postinfection (p.i.) (25, 28, 32). LDV infection is associated with impaired clearance of plasma lactate dehydrogenase, as well as several other enzymes, and increased plasma LDH serves as a marker for LDV infection. Acute infection is invariably followed by a life-long persistent infection, characterized by continued elevation of plasma enzyme levels and a chronic low-level viremia associated with virus-antibody complexes (22, 23). LDV infection results in modifications of the host immune response by mechanisms not yet understood. The humoral antibody response to some heterologous T cell-dependent antigens, such as human immunoglobulin G (IgG), has been reported to be enhanced during the acute and chronic stages of infection (14, 20, 24, 27), and the enhancement seems to reflect mainly an increase in IgM antibody (27). The humoral antibody responses to other antigens, on the other hand, are not affected by LDV infection, or are only enhanced during the acute phase of infection, and may even be slightly depressed during the chronic stage (14, 20, 24, 27). In contrast to the adjuvant effects of LDV infection on the humoral antibody response, the cellular immune response towards transplants is depressed during the acute phase of infection, but is generally normal in chronically infected mice (12, 13, 19).

Infection with LDV provides an interesting model system for the study of mechanisms of viral persistence and virus effects on the host immune system. In this paper, we present the results of our studies on the immune response to LDV in several strains of mice, using a sensitive enzyme-linked immunosorbent assay (ELISA) developed to detect specific antiviral antibody.

Furthermore, we provide evidence that persistent LDV infection causes an increase in total plasma IgG and this increase is a result of polyclonal stimulation of IgG synthesis.

(Some of these results have been reported in preliminary form [W. A. Cafruny and P. G. W. Plagemann, Fed. Proc. 39:781, 1980].)

MATERIALS AND METHODS

Mice. Female mice were either bred in our mouse colony or purchased from the following sources: outbred Swiss mice from BioLabs, St. Paul, Minn.; normal BALB/c and BALB/c homozygous (nu/nu) athymic mice from Sprague Dawley, Madison, Wis.; and Swiss homozygous (nu/nu) athymic mice from Charles River Breeding Labs, Wilmington, Mass.

Collection of mouse plasma. Plasma for biochemical or immunological assays was obtained by retroorbital sinus bleeding of mice into heparinized capillary tubes. Blood cells were separated by centrifugation at 500 × *g* for 15 min, and plasma samples were used either fresh or stored at −20°C.

LDV. Our strain of LDV was originally isolated from a C3H mouse carrying a transplantable tumor (3). LDV titers were determined by an endpoint dilution assay in mice (28). Virus stocks of standard virus consisted of plasma pools from 18- to 20-h LDV-infected Swiss mice diluted twofold with minimum essential medium and contained about 10^9 ID_{50}/ml (3, 35).

Purification of LDV. LDV from pooled plasma of 100 to 200 18- to 20-h LDV-infected mice was collected by centrifugation through a 25% sucrose cushion for 5 h at 24,000 rpm in a Beckman SW27 rotor. The pelleted virus was suspended in 0.15 M phosphate-buffered saline (PBS; pH 7.4) and centrifuged on a continuous (0.5 to 1.5 M) sucrose density gradient at 22,000 rpm for 18 h (3). The virus-containing gradient fractions were pooled, and the virus was repelleted and suspended in PBS. The suspension contained approximately 10^{11} ID_{50}/ml and was stored at −70°C until used.

Isolation of LDV-IgG complexes. Plasma from mice chronically infected with LDV was diluted 2:1 with PBS (pH 7.4) and passed through a column (diameter, 0.7 cm; length, 7 cm) containing protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was washed extensively with PBS, bound material was eluted with 0.15 M citrate-phosphate buffer (pH 3.1), and the protein A eluate was immediately neutralized with 1 N NaOH. The total amount of IgG present in the plasma was reduced by >90% by this technique.

Assay of plasma IgG. The concentration of total mouse IgG in plasma was determined by the technique of radial immunodiffusion (Miles Laboratories, Inc., Elkhart, Ind.).

Quantitation of mouse anti-LDV IgG. Mouse antibody to LDV was measured by an ELISA technique (9). Purified LDV (2×10^9 ID_{50}) in 0.2 ml of carbonate coating buffer (pH 8.6) was added to the wells of 96-well microtiter plates, and the plates were stored at 4°C for 18 h. After being washed three times with PBS-Tween 20 (pH 7.4), various dilutions of immune or normal mouse plasma, made in PBS-Tween, were added in 0.2-ml volumes to the virus-coated microtiter wells, and the plates were incubated at 25°C for 2 h. After being washed three more times with PBS-Tween, all wells received 0.2 ml of alkaline phosphatase-conjugated goat anti-mouse IgG specific for the Fab fragment of mouse IgG (Polysciences, Inc., Warrington, Pa.) diluted 1:175 in PBS-Tween. After a 2-h incubation period at 25°C and three washes with PBS-Tween, 0.2 ml of a solution of phosphatase substrate (Sigma Chemical Co., St. Louis, Mo.; 104; 1 mg/ml) was added per well. After 30 min of incubation at 25°C, the reaction was stopped by the addition of 50 μl of 1 N NaOH. The absorbancy of the contents of each well was measured at 400 nm. All values are averages of duplicate determinations.

Purification of mouse IgG. Pooled plasma from 50 to 100 mice was chromatographed on protein A-Sepharose CL-4B as described above. The eluted IgG was then filtered through a Sephadex G-200 column equilibrated with citrate-phosphate buffer (pH 3.1), and 1-ml fractions were collected at 4°C. The single major protein peak of IgG was pooled and neutralized with 1 N NaOH. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that >90% of the total protein consisted of heavy and light IgG chains.

Isoelectric focusing. Samples of purified mouse IgG were electrofocused with an LKB 2117 Multiphor (Stockholm, Sweden) thin-layer apparatus according to the manufacturer's specifications (38). Samples containing between 14 and 27 μg of IgG were electrofocused for 4 h through a pH gradient of 3.5 to 9.5, and protein bands were stained with Coomassie blue.

RESULTS

Isolation of LDV-IgG complexes. We have utilized the high affinity of staphylococcal protein A for mouse IgG (10, 11) as a means of isolating LDV bound to IgG. When pooled plasma from mice infected with LDV for 2 to 4 months was passed through a column of protein A-Sepharose CL-4B, practically all LDV infectivity was adsorbed (Fig. 1). About 20% of the total infectious units were recovered upon elution of the bound IgG with buffer (pH 3.1). The loss of infectivity during the isolation process was not surprising because of the acid lability of LDV (32). In contrast, <0.01% of LDV infectious units from plasma of 24-h-infected mice was reversibly bound to a protein A column. The results show that LDV is present in the plasma of chronically infected mice almost entirely as an infectious complex with IgG.

Reactivation of LDV from the neutralized state. IgG-LDV complexes isolated from protein A-Sepharose or noncomplexed LDV obtained from the plasma of acutely infected mice were incubated in PBS containing 6.4 mM 2-mercaptoethanol (2ME) at 4°C for 2 h. Table 1 shows representative data demonstrating that treatment with 2ME caused some inactivation of noncomplexed LDV, whereas it increased by 1 to 2 logs the infectivity titer of IgG-LDV complexes. Thus, reduction of IgG-LDV complexes

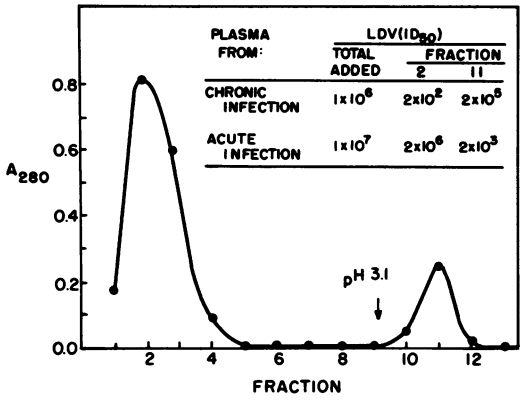

FIG. 1. Chromatography of plasma from acutely and persistently LDV-infected Swiss mice on protein A-Sepharose CL-4B. A total of 1.0 ml of pooled plasma from 2- to 4-month-LDV-infected Swiss mice was placed on the column, and the column was washed with PBS. Adsorbed material was eluted with citrate-phosphate buffer, pH 3.1. Fractions of 1 ml were collected and analyzed for absorbance at 280 nm and LDV by titration in mice. Plasma from 20-h-infected mice was chromatographed in the same manner (profile not shown). The LDV infectivity units placed on the columns and recovered in the peak fractions are summarized in the insert. Total IgG concentrations for the fractions shown were: precolumn, 6 mg/ml; fraction 2, 0.4 mg/ml; fraction 11, 5.4 mg/ml. A_{280}, Absorbance at 280 nm.

with 2ME results in reactivation of a previously neutralized fraction of the virus and indicates that immune-complexed virus is partially neutralized and that this state of neutralization is reversible.

Immune response to LDV. Swiss, BALB/c normal, and BALB/c nude (nu/nu) mice were bled at various times after infection with LDV, and their plasma, at a 1:400 dilution, was assayed for specific anti-LDV IgG by the ELISA method. Preliminary experiments (5) had shown that specific anti-LDV IgG could readily be detected in the plasma of a 4-month-LDV-infected Swiss mouse at dilutions of 1:400 to 1:800. The results of a representative experiment (Fig. 2) demonstrate that mice of all three strains began producing anti-LDV IgG between 1 and 3 weeks p.i. The Swiss mouse produced antibody in greatest quantity, but the nude BALB/c mouse produced nearly as much antibody to LDV as the wild-type BALB/c mouse. Overall, we compared the anti-LDV plasma antibody of a total of six individual nude mice (three BALB/c and three Swiss) with age-matched wild-type controls and found that nude mice responded with a mean antibody level of 45% of that of control mice (range, 15 to 58%).

Plasma IgG levels during LDV infection. Total plasma IgG levels were measured in both normal and nude BALB/c and Swiss mice during LDV infection. Figure 3 shows the results of these experiments, which demonstrate that the total plasma IgG levels of normal, as well as nude, mice increased progressively during LDV infection. In particular, it should be noted that the normally low plasma concentrations of total IgG in nude mice (1 mg/ml or less) increased to the level found in thymically normal mice. Total plasma IgG concentrations of age-matched uninfected control (+/+) or (nu/nu) mice remained within 10% of the base-line values during the experiments shown in the figure.

TABLE 1. Effect of 2ME on LDV infectivity of LDV-IgG complexes and plasma of acutely infected mice[a]

LDV	Expt	LDV (ID_{50}/ml)	
		Untreated	2ME-treated
IgG-LDV	1	1×10^4	1×10^5
	2	1×10^3	1×10^5
Standard LDV	1	1×10^9	1×10^8
	2	1×10^7	5×10^6

[a] Samples of IgG-LDV complexes isolated on protein A-Sepharose or noncomplexed LDV obtained from plasma of acutely infected mice (standard LDV) were incubated with and without 6.4 mM 2ME at 4°C for 1 h and then titrated in mice. Standard LDV consisted of gradient-purified LDV diluted 1:100 in PBS (experiment 1) or plasma from mice 18 h p.i. with LDV and diluted 1:20 in PBS (experiment 2).

Protein A-binding fraction of LDV in plasma from nude mice. Because of the presence of specific anti-LDV IgG and increased total plasma IgG levels in chronically infected nude mice, we measured the fraction of LDV in the plasma of these mice that bound to protein A. Because of the limited amount of plasma available, we used a batch method. Table 2 shows that the binding of LDV-IgG complexes to protein A in the batch process was not as efficient as in the columns (Fig. 1), but that, nevertheless, at least 50% of the LDV infectivity from the plasma of chronically infected nude mice did bind and was eluted by buffer (pH 3.1), just as from the plasma of chronically infected Swiss mice, whereas plasma LDV from acutely infected nude mice failed to bind protein A.

Isoelectric focusing pattern of IgG from LDV-infected mice. IgG prepared by affinity chromatography on protein A-Sepharose CL-4B followed by gel filtration under acid-dissociating conditions was analyzed by isoelectric focusing. A comparison of IgG from persistently LDV-infected and control uninfected Swiss mice showed the following. First, purified IgG from both uninfected and persistently infected mice yielded a total of 16 corresponding fractions, all of which were present in greater amounts in

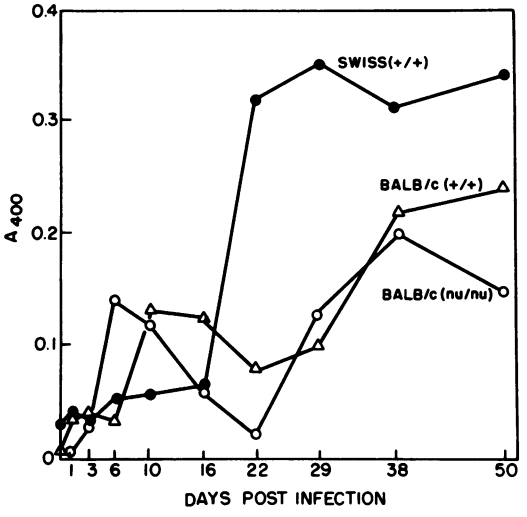

FIG. 2. Formation of anti-LDV IgG in LDV-infected mice determined by ELISA method. Plasma samples were obtained from individual BALB/c (+/+), Swiss (+/+), and BALB/c (nu/nu) mice at the indicated times p.i. with 10^6 ID_{50} of LDV per mouse. The plasma was diluted 1:400 in PBS-Tween and analyzed by the ELISA. All values are averages of duplicate determinations and have been corrected for absorbance observed using a 1:400 dilution of plasma from uninfected mice. A_{400}, Absorbance at 400 nm.

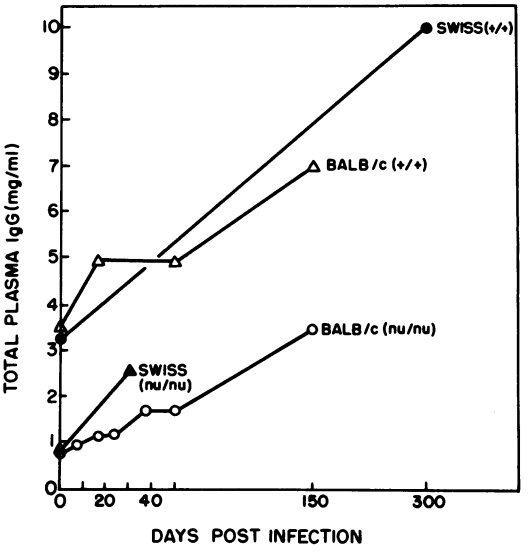

FIG. 3. Total plasma IgG elevation during LDV infection. At various times after infection with 10^6 ID_{50} of LDV per mouse, plasma samples were analyzed for total IgG by radial immunodiffusion. The values shown for BALB/c (+/+) and BALB/c (nu/nu) mice are for the same plasma samples used for anti-LDV IgG assays in the experiment shown in Fig. 2. The values for Swiss (nu/nu) mice represent a mean of four separate mice, and those for Swiss (+/+) mice represent a pool of plasma from 50 mice. Total IgG levels in age-matched control mice remained within 10% of the day 0 values shown for all strains of mice during the experiments.

plasma from infected than from uninfected mice. Second, IgG from persistently infected mice yielded two unique bands focusing at about pH 5.9 and 6.4, which were not obtained from IgG of uninfected mice. These bands may represent anti-LDV IgG, or alternatively, they may result from an enhanced synthesis of normal IgGs, which are inapparent in the sample from uninfected mice due to their normally low concentration. Some of the bands were not sufficiently intense for clear photographic reproduction, and the patterns are, therefore, not shown.

DISCUSSION

Present evidence indicates that persistent infection by LDV is maintained by a susceptible subpopulation of macrophages, which produces virus at a level sufficient to infect newly permissive host cells as they arise (35, 36). The results of the present investigation confirm the finding (23) that in the persistent stage of LDV infection, virus is present in the plasma in the form of infectious IgG-LDV complexes and show that these complexes account for over 99% of the circulating infectious virus (Fig. 1). These results are consistent with the finding that LDV titers in serum from persistently infected mice can be substantially reduced by incubation with anti-mouse IgG or IgA, but not anti-IgM (22). Our present data demonstrate that, in addition to being intrinsically infective, LDV-IgG complexes can be significantly reactivated from a state of partial neutralization by treatment with 2ME (Table 2). The mechanisms of partial neutralization of LDV by IgG and of reactivation from neutralization by 2ME are not known. Partial neutralization probably not only reflects the aggregation of virus particles by antibody (22, 32), but may also result from a reversible conformational change in the virion induced by IgG binding which interferes with the infectious process. Regardless, reactivation by 2ME treatment is probably related to the reduction of interchain or intrachain disulfide bonds of IgG molecules and disruption of the complexes (6, 8). Others have reported that poliovirus neutralized by F $(ab')_2$ fragments of antibody to virus also was reactivated by treatment with 2ME (15).

Incomplete neutralization of LDV infectivity by mouse IgG may contribute to the ability of this virus to persist in mice in the face of a vigorous host immune response. Other persistent viruses are also known to circulate in com-

TABLE 2. Protein A binding of LDV complexes in plasma from persistently infected nude mice[a]

Swiss mice	Time p.i.	Total LDV (ID_{50})		
		Control	Post-adsorption	Protein A eluate
Nude	2 months	1×10^5	5×10^3	5×10^3
Wild type	2 months	1×10^5	1×10^4	1×10^4
Nude	20 h	1×10^5	5×10^4	1×10^1

[a] Samples of 0.2 ml of plasma or a dilution thereof containing the indicated amounts of LDV (control) were mixed with 0.25 ml of a 50% (wt/vol) suspension of protein A-Sepharose CL-4B, and the mixture was incubated at 25°C for 10 min. The Sepharose beads were pelleted by centrifugation, and titers of the supernatant were determined for LDV (postadsorption). The pellet was washed three times with PBS and then mixed with 0.2 ml of buffer (pH 3.1). After centrifugation, titers of the supernatant (eluate) were determined for LDV.

plexes with IgG in which virus infectivity is incompletely neutralized (17, 26, 29, 31). Because LDV replication seems limited to macrophages (2) and these cells possess Fc receptors, the binding of IgG to LDV may aid rather than prevent the infection of newly generated permissive macrophages during the persistent stage. In fact, we have demonstrated that when LDV is present at a low multiplicity of infection, IgG enhances its infectivity for primary macrophage cultures up to 100-fold (5).

Previous studies have demonstrated anti-LDV antibody in mice by measuring virus neutralization (23, 33) or immunofluorescence (30). In the present work, we developed an ELISA which is specific and sensitive in detecting and quantitating anti-LDV antibody (Fig. 2). We found that plasma anti-LDV IgG is first detectable as early as 6 days p.i. Fluorescent antibody to LDV was also first detected about 6 days p.i. (30), and LDV infection of germfree mice resulted in an increase in IgG, beginning 6 days p.i. (24), but to what extent this IgG represented anti-LDV antibody was not determined. In contrast, LDV-neutralizing antibody was first detectable at only 1 to 3 months p.i. (23, 33). It seems likely, therefore, that most of the early anti-LDV IgG represents antibody to sites on the virion not critical for neutralization or low-affinity antibody which is nonneutralizing.

We found that considerable amounts of anti-LDV IgG were produced in all three mouse strains investigated throughout the 50-day period of the experiment, with the highest antibody levels occurring in outbred Swiss mice. Of particular interest is the finding that athymic mice (nu/nu) exhibited anti-LDV IgG levels almost as high as those of wild-type BALB/c mice (Fig. 2), since the IgG immune response to viral antigens, including those of togaviruses, has generally been found to be thymus dependent (4, 37).

The unusual response of nude mice to LDV infection might be related to the general stimulation of IgG production induced by this virus (Fig. 3). The IgG levels of uninfected nude mice were very low, as reported by others (16, 34), but increased during 150 days of infection with LDV to about the level present in uninfected BALB/c mice. Isoelectric focusing of purified IgG demonstrated that all 16 normal plasma IgG fractions separated by this technique were increased in persistently LDV-infected BALB/c mice. This finding indicates that LDV infection results in polyclonal activation of IgG synthesis. Another mechanism which could account for the progressive increase in IgG levels in chronically infected mice might be an impaired clearance of IgG or IgG-antigen complexes. This mechanism, however, seems to be ruled out by the finding that the rate of clearance of ^{131}I-labeled mouse IgG was about the same in uninfected and LDV-infected mice during the first 11 days after infection (24).

It seems likely that polyclonal lymphocyte activation also accounts for the increase in total plasma IgG in nude mice because of the magnitude of the IgG increase and because both total plasma IgG and anti-LDV IgG increase during the persistent stage, but so far we have not had sufficient purified IgG from these mice for further study.

Another question is whether the stimulation of IgG synthesis in nude mice by LDV infection bypasses a requirement for T cell helper functions or results from the differentiation of precursor T cells in the absence of a thymus. It is possible, for example, that LDV could affect lymphocyte functions indirectly by a modification of macrophages in infected animals. We found that acute infection results in a marked, but transient, enhancement of the Fc and C3 receptor activity of peritoneal macrophages which seems to be interferon mediated (21). On the other hand, one could envision that lymphocyte functions might be affected by the continuous destruction of the LDV-permissive subpopulation of macrophages in persistently infected animals (35, 36). In this connection, it is also noteworthy that thymectomized mice infected at birth with LDV have been reported to live longer than noninfected mice (7) and that interferon production is induced by lymphocytic cho-

riomeningitis virus to a 10-fold greater extent in nude than in wild-type BALB/c mice (18).

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