INFECTIOUS VIRUS–ANTIBODY COMPLEX IN THE BLOOD OF CHRONICALLY INFECTED MICE*

By ABNER LOUIS NOTKINS, M.D., SUELLEN MAHAR, CHRISTINA SCHEELE,‡ AND JOEL GOFFMAN§

(From the Laboratory of Microbiology, National Institute of Dental Research, United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, Maryland)

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In mice, lactic dehydrogenase virus (LDV) acts as an immunologic adjuvant in that it enhances the capacity of infected animals to produce antibody to a foreign protein (human γ-globulin) (1). However, attempts to demonstrate neutralizing antibody to the virus in the blood of mice, rabbits, and guinea pigs have been unsuccessful (1–4). Testing for antiviral antibody in mice was particularly difficult because of the lifelong viremia and the consequent presence of infectious virus in the sera of chronically infected animals (1).[1] The experiments reported herein show that (*a*) if measures are first taken to inactivate the infectious virus in the chronic sera, neutralizing antibody can be demonstrated, and (*b*) the infectious virus which persists in the circulation of chronically infected mice after the appearance of anti-LDV exists in the form of an infectious virus-antibody complex which is relatively resistant to further neutralization.

Materials and Methods

Mice.—CAF-1 male and female mice were used throughout these experiments. Mice 4 to 6 wk old were used routinely to titer the virus.

Antibody to Normal Mouse Sera and Normal Mouse γ-Globulin.—Goat anti-mouse sera (lot 71-227), goat anti-mouse γ-globulin (lot 70898), and goat anti-human γ-globulin were obtained from Hyland Laboratories, Los Angeles. Normal goat sera were obtained from the animal farm of the National Institutes of Health and from Pentex Laboratories, Kankakee, Illinois. Rabbit anti-mouse sera was prepared by Dr. R. T. Evans of the National Institute of Dental Research by injecting rabbits intravenously with normal mouse sera 3 times a week for 3 wk. Sera was collected 10 days after the last injection.

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‡ Present Address: Department of Bacteriology and Immunology, Harvard Medical School, Boston.

§ Summer participant (1965) in the Commissioned Officer Student Training and Extern Program of the United States Public Health Service, Department of Health, Education, and Welfare. Present Address: University of Maryland School of Medicine, Baltimore.

[1] Hereinafter referred to as "chronic sera" in the interest of brevity.

Virus.—The preparation of LDV and the virus assay were performed as described previously (5), except that each virus dilution was injected into 10 mice instead of 5. Unless indicated otherwise Eagle's basal medium with 20% veal infusion broth (EBMV) was used as the diluent. Acute virus pools (referred to as stock virus) were obtained from mice that had been infected for 24 hr. Chronic virus pools were obtained from mice that had been infected for 3 or more months. The titer of the acute pools was approximately $10^{10.0}$ ID_{50}/ml, while the titer of the chronic pools varied between $10^{4.0}$ to $10^{5.0}$ ID_{50}/ml.

Antibody to Virus.—Before the sera from LDV-infected mice could be tested for neutralizing antibody it was necessary to render the sera noninfectious. This was done by inactivating the virus with ether (6). Serum or plasma was collected from mice at various intervals after injection of the virus, pooled, and diluted with an equal volume of Eagle's basal medium (EBM). The diluted serum was then shaken with an equal volume of fresh ethyl ether (Mallinckrodt Chemical Works, St. Louis) at room temperature for 3 min. The aqueous phase was removed and shaken with ether three more times. Residual ether was removed by aeration with nitrogen. Serum or plasma from uninfected mice was prepared in the same way. Following treatment all preparations were shown to be noninfectious and were stored until needed in aliquots of 0.8 ml at −55°C. The fact that a number of different pools of anti-LDV were prepared during the course of this work and that these pools were collected at various intervals after initiation of the infection might account for the differences in the degree of viral neutralization noted between experiments. Unless indicated otherwise, the terms anti-LDV and normal mouse sera refer to materials that had been pretreated with ether. The volume of each is expressed in terms of the original undiluted sera.

Virus Neutralization.—Neutralization of the virus was carried out by incubating a constant volume of anti-LDV (0.3 ml) at 37°C for 1 hr with serial tenfold dilutions of the stock virus in a final volume not exceeding 1.2 ml. Reaction mixtures containing normal mouse sera were employed as controls in each experiment. Following incubation the reaction mixtures were immediately placed in ice and 0.1 ml of each mixture was injected intraperitoneally into each of 10 mice. The number of mice that became infected at each dilution was determined and the ID_{50}/ml was calculated by the method of Reed and Muench (7). The difference in end points between the control and test sera was expressed in terms of a neutralization index (8). A difference of less than 0.7 log units was considered negative, 0.7 to less than 1.0 log units was equivocal, 1.0 to less than 1.5 log units as positive, and over 1.5 log units as strongly positive.

In other neutralization experiments, 0.3 ml of antisera was added to a known virus concentration in a final volume not exceeding 1.5 ml and incubated at 37°C for 1 hr. In each experiment virus incubated with normal mouse sera served as the control. Immediately following incubation the reaction mixtures were diluted serially in tenfold steps in ice cold EBMV. Animals were injected intraperitoneally with 0.1 ml of each of the appropriate dilutions, the number of animals that became infected was determined, and the virus titer was calculated.

Virus Sensitization.—The term virus sensitization, as used throughout this paper, refers to the attachment of mouse anti-LDV to LDV without concomitant loss of infectivity. As reported below, our experiments showed that sensitization made the virus susceptible to neutralization by anti-mouse sera or anti-mouse γ-globulin. The unsensitized virus was totally resistant to neutralization by these reagents. To test for sensitization, undiluted goat anti-mouse sera (0.2 ml) or undiluted rabbit anti-mouse sera (0.3 ml) was incubated at 37°C for 1 hr with a known virus concentration in a final volume not exceeding 1.8 ml. Reaction mixtures containing normal goat or rabbit sera served as controls in each experiment. Serial tenfold dilutions of the reaction mixtures were then prepared and 0.1 ml of each dilution was injected intraperitoneally into each of 10 mice. A reduction in virus titer of over 1.0 log units as compared to the controls was taken as positive evidence of virus sensitization.

In other experiments a constant volume of goat anti-mouse sera (0.2 ml) was incubated

at 37°C for 1 hr with serial tenfold dilutions of the virus in a final volume not exceeding 1.2 ml. Following incubation 0.1 ml of each dilution was injected intraperitoneally into each of 10 mice and the neutralization index was calculated (7, 8).

RESULTS

Demonstration of Antibody to LDV.—Mice were infected with LDV and at various times thereafter sera were collected and treated with ether to inactivate residual infectious virus. Sera from normal mice were collected and prepared in the same way. Both sera were then incubated with serial tenfold dilutions of the stock virus and the neutralization index was determined. The experiments summarized in Table I failed to reveal any neutralizing antibody during the first month of the infection. However, at about 10 wk a small amount of antibody seemed to be present. At 13 wk and persisting thereafter neutralizing antibody was readily detected.

TABLE I

*Time of Appearance of Anti-LDV**

Post-LDV	Anti-LDV‡
wk	
0	0
2	0
3	0
4	0
10	±
13	+
16	++
31	++

* At the times indicated, sera from infected and uninfected mice were collected and treated with ether as described under Materials and Methods. Each sera was then incubated with serial tenfold dilutions of the stock virus and the neutralization index (NI) was determined.

‡ 0 = negative, NI less than 0.7 log units; ± = equivocal, NI 0.7 to less than 1.0 log units; + = positive, NI 1.0 to less than 1.5 log units; and ++ = strongly positive, NI over 1.5 log units.

To see if anti-LDV could be demonstrated as readily if the infectious virus in the chronic sera was inactivated by ultraviolet light (UV) rather than ether, the following experiment was performed. Normal mouse sera and sera from mice that had been infected with LDV for about 7 months were exposed to UV as described previously (1). Each sera was then incubated at 37°C for 1 hr with approximately $10^{5.5}$ ID_{50}/ml of stock virus. The reaction mixtures were then serially diluted and the virus titer was determined. As seen in Table II, the anti-LDV activity in sera prepared by the UV method was only slightly lower than that found in sera prepared by the ether method. Normal sera that had been exposed to UV or ether had no deleterious effect on the virus.

To demonstrate neutralizing antibody without subjecting the sera to UV or ether treatment, the following experiment was performed. Sera from mice that had been infected with LDV for 3 months was diluted with an equal volume of EBM. One-half was treated as usual with ether while the other half was left untreated. Each preparation was then incubated with approximately

TABLE II

Comparison of Anti-LDV Activity in UV and Ether-Treated Sera

Treatment of sera	Virus titer ID_{50}/ml (log 10)*	
	Normal sera	Anti-LDV‡
None	5.3	ND§
UV	5.5	3.7
Ether	5.3	3.1

* Approximately $10^{5.5}$ ID_{50}/ml of the stock virus was incubated with each of the sera at 37° C for 1 hr. The reaction mixtures were then serially diluted and the virus titer was determined.

‡ Sera from mice that had been infected with LDV for 7 months.

§ Not done.

TABLE III

*Anti-LDV Activity in Ether-Treated and Untreated Sera**

Sera	Treatment	Virus titer ID_{50}/ml (log 10)
Uninfected‡	None	7.7
Infected§	None	6.6
Infected§	Ether	6.5

* Approximately $10^{7.7}$ ID_{50}/ml of the stock virus was incubated with each sera at 37°C for 1 hr. The reaction mixtures were then serially diluted and the virus titer was determined.

‡ Sera from normal mice.

§ Sera from mice that had been infected with LDV for 3 months.

$10^{7.7}ID_{50}$/ml of the stock virus at 37°C for 1 hr. Serial dilutions were performed and the virus titer was determined. Since sera from the chronically infected mice contained approximately $10^{4.0}ID_{50}$/ml, whereas the concentration of the stock virus in the reaction mixture was approximately $10^{7.7}ID_{50}$/ml, the virus in the untreated chronic sera would be diluted out before the stock virus in the reaction mixture. This made it possible to measure the anti-LDV activity in the untreated sera. The data in Table III show that the untreated sera neutralized the virus to the same extent as the ether treated sera.

Neutralization Conditions and Properties of Anti-LDV.—To determine the optimal in vitro conditions for virus neutralization, the virus stock was diluted

to contain approximately $10^{4.7}$ ID_{50}/ml and was incubated with anti-LDV under different time and temperature conditions. Following incubation the reaction mixtures were serially diluted and the virus titer was determined. As seen in Table IV, incubation with anti-LDV at 37°C for 1 hr decreased the virus titer by 1.0 log units. Incubation at 37°C for 180 min decreased the virus titer by 1.5 log units, but, as indicated by the loss in virus titer in the control tube, the prolonged incubation had resulted in substantial thermal inactivation of the virus. No detectable inactivation occurred at 37°C for 15 min or at 0°C for 60 min. However, other experiments showed that when more potent prepara-

TABLE IV

*Effect of Time and Temperature on Virus Neutralization**

Incubation conditions		Virus titer ID_{50}/ml (log 10)	
		Incubated with	
Time	Temperature	Normal Sera	Anti-LDV‡
min	°C		
15	37	4.7	4.7
60	37	4.5	3.5
60	0	4.7	>4.7
180	37	3.8	2.3

* Approximately $10^{4.7}$ ID_{50}/ml of the stock virus was incubated with anti-LDV. The reaction mixtures were serially diluted and the virus titer was determined.

‡ Sera from mice that had been infected with LDV for 4 months.

tions of anti-LDV were employed, neutralization could be detected within 15 min at 37°C and within 60 min at 0°C.

Although anti-LDV was capable of neutralizing nearly 99% of the stock virus within 1 hr at 37°C (Table II), approximately 1.0% of the virus remained infectious. To see whether the amount of antibody in the reaction mixture was the limiting factor responsible for the residual infectious virus, the following experiment was performed (Table V). Approximately $10^{5.0}$ ID_{50}/ml was incubated with normal sera (tubes A and C) or anti-LDV (tubes B and D) at 37°C. At the end of 1 hr an additional $10^{5.0}$ ID_{50}/ml was added to tubes C and D, while only diluent was added to tubes A and B. The reaction mixtures were then incubated for another hour at 37°C, serially diluted, and the virus titer was determined. The data in Table V show that anti-LDV neutralized about 99% of the original virus (tubes A and B). When the second aliquot of virus was added (tubes C and D), again approximately 99% of the virus was neutralized. The results of these experiments indicate that although 1.0% of the original virus was not neutralized, anti-LDV was in excess in the original reac-

tion mixture. The nature of this "resistant fraction" will be considered in more detail later in this paper.

In the neutralization experiments described above, the amount of anti-LDV was always kept constant (0.3 ml) while the concentration of virus was varied between experiments. To study the effect of different concentrations of anti-LDV on the neutralization of a constant amount of virus, anti-LDV was serially diluted and then incubated with $10^{2.0}$ ID_{50}/ml. A 1 in 10 dilution of anti-LDV was found to produce a substantial reduction in the neutralizing activity of the sera. The possibility that low levels of an essential cofactor, such as comple-

TABLE V

Demonstration of Excess Antibody in Reaction Mixture

Incubation tubes*	Incubation I	Incubation II	Virus titer ID_{50}/ml (log 10)
A	LDV plus normal sera	Diluent only	4.6
B	LDV plus anti-LDV‡	Diluent only	2.5
C	LDV plus normal sera	Diluent plus LDV	5.4
D	LDV plus anti-LDV‡	Diluent plus LDV	2.9

* Approximately $10^{5.0}$ ID_{50}/ml of the stock virus was incubated at 37°C with normal mouse sera or anti-LDV (incubation I). At the end of 1 hr an additional $10^{5.0}$ ID_{50}/ml was added to tubes C and D while only diluent (EBMV) was added to tubes A and B (incubation II). All tubes were incubated for another hour at 37°C, serially diluted, and titered.

‡ Sera from mice that had been infected with LDV for 5 months.

ment, are involved in the neutralization reaction is presently under investigation.

Other properties of anti-LDV were studied by subjecting the antibody to various types of treatment and then incubating it with approximately $10^{1.5}$ ID_{50}/ml of stock virus at 37°C for 1 hr. Each reaction mixture was then injected into mice and the neutralizing activity of the antisera was calculated by subtracting the percentage of mice that became infected from 100. As seen in Table VI, anti-LDV that had been stored at −55°C for 1 month or that had been dialyzed against saline for 6 hr retained its neutralizing activity. However, considerable loss of activity occurred when anti-LDV was heated at 56°C for 30 min. Anti-LDV that had been pretreated at 37°C for 30 min with goat anti-mouse sera or goat anti-mouse γ-globulin also showed a substantial loss in neutralizing activity.

Virus Sensitization in Vivo.—The presence of both anti-LDV and infectious virus in the same serum pointed to the possibility that some antibody molecules might be attached to the infectious virus particle (sensitization). The reaction of anti-mouse sera or anti-mouse γ-globulin with anti-LDV (Table VI) suggested that if anti-LDV were attached to the virus particle

then anti-mouse sera might also react with and possibly neutralize the infectious LDV–anti-LDV complex. To examine this possibility, virus obtained from mice after the appearance of circulating anti-LDV was incubated with goat anti-mouse sera or goat anti-mouse γ-globulin. Goat anti-human γ-globulin and normal goat sera served as controls. Following incubation, the reaction mixtures were serially diluted and the virus titer was determined. As seen in Table VII, anti-mouse sera and anti-mouse γ-globulin both reduced the virus titer while anti-human γ-globulin had no effect.

TABLE VI

Neutralizing Activity of Anti-LDV Following Various Types of Treatment

Mouse sera	Treatment	Neutralizing activity*
		(%)
Anti-LDV‡	Untreated	100
Anti-LDV	Stored at −55°C/1 month	100
Anti-LDV	Dialyzed for 6 hr§	90
Anti-LDV	Heated at 56°C/30 min	40
Anti-LDV	Incubated with anti-mouse γ-globulin‖	60
Anti-LDV	Incubated with anti-mouse sera‖	20
Normal	Untreated	0

* Anti-LDV was treated in various ways and then incubated at 37°C for 1 hr with approximately $10^{1.5}$ ID_{50}/ml of the stock virus. Each reaction mixture was then injected into mice and the neutralizing activity was calculated by subtracting the percentage of mice that became infected from 100.

‡ Sera from mice that had been infected with LDV for 4 months.

§ Dialyzed against saline at 4°C.

‖ Anti-LDV (0.3 ml) was incubated at 37°C for 30 min with goat anti-mouse sera or goat anti-mouse γ-globulin (0.3 ml) prior to incubation with the virus.

If, as the above experiment suggested, the goat anti-mouse sera was reacting with the anti-LDV that was attached to the virus particle, then virus obtained from animals before anti-LDV appeared in the circulation would not be sensitized and thus not react with the goat anti-mouse sera. To test this hypothesis, virus obtained from mice before and after the appearance of anti-LDV was serially diluted and incubated with goat anti-mouse sera or normal goat sera and the ID_{50}/ml for each set of dilutions was determined. As seen in Table VIII, goat anti-mouse sera had no effect on the virus obtained from mice prior to the appearance of anti-LDV, whereas it produced a substantial reduction in the titer of the virus obtained from mice after the appearance of anti-LDV. Similar results were obtained with an anti-mouse sera prepared in rabbits.

To study in more detail the time at which sensitized virus appeared in the circulation of chronically infected mice, virus pools were collected from mice at various intervals post-LDV. Serial tenfold dilutions of each pool were then

incubated with either goat anti-mouse sera or normal goat sera and the neutralization index was determined. As seen in Table IX, evidence of virus sensitization was not apparent until 11 wk post-LDV. Unequivocal sensitization

TABLE VII

*Effect of Anti-Mouse γ-Globulin and Anti-Mouse Sera on Titer of Sensitized Virus**

Sera (goat)	Virus titer ID_{50}/ml (log 10)
Normal	2.8
Anti-human γ-globulin	2.7
Anti-mouse γ-globulin	2.0
Anti-mouse sera	1.4

* Virus obtained from mice after the appearance of circulating anti-LDV (3 months post-LDV) was diluted to contain approximately $10^{2.8}$ ID_{50}/ml and was incubated at 37°C for 1 hr with the various goat sera. The reaction mixtures were then serially diluted and the virus titer was determined.

TABLE VIII

Effect of Anti-Mouse Sera on Titer of Sensitized and Unsensitized Virus

Sera	Titer of virus ID_{50}/ml (log 10)*	
	Unsensitized virus‡	Sensitized virus§
Goat		
Normal	2.6	2.4
Anti-mouse	2.6	<1.0
Rabbit		
Normal	2.6	2.2
Anti-mouse	2.4	<1.0

* The virus pools were diluted to contain approximately $10^{2.6}$ ID_{50}/ml. Serial tenfold dilutions were then incubated at 37°C for 1 hr with goat or rabbit sera and the ID_{50}/ml (log 10) for each set of dilutions was determined.

‡ Obtained from mice prior to the appearance of circulating anti-LDV (2 wk post-LDV).

§ Obtained from mice after the appearance of circulating anti-LDV (4 months post-LDV).

was detected at 15 wk. These times correspond closely with the appearance of circulating anti-LDV (Table I).

If the goat anti-mouse sera were reacting with the anti-LDV that was attached to the virus particle then one would not expect the unsensitized progeny of sensitized virus to react with anti-mouse sera. To see if this were the case, sensitized virus was diluted $10^{-3.0}$ and 0.1 ml was injected intraperitoneally into recipient mice. 15 hr later the viral progeny was harvested and

incubated in the usual way with anti-mouse sera. As seen in Table X, the progeny failed to react with anti-mouse sera.

Virus Sensitization in Vitro.—The following experiment was designed to see if unsensitized virus could be sensitized in vitro by incubation with anti-LDV. As outlined in Table XI, approximately $10^{8.0}$ID$_{50}$/ml of unsensitized stock virus was incubated with normal mouse sera (groups A and B) or anti-LDV (groups C and D) at 37°C for 1 hr. Immediately following incubation the reaction mixtures were diluted $10^{-3.2}$ in ice cold EBMV. To aliquots of the latter dilution, normal goat sera or goat anti-mouse sera was added and allowed to incubate at 37°C for 1 hr (incubation II). Each reaction mixture was then serially

TABLE IX

Time of Appearance of Sensitized Virus

Post-LDV*	Sensitized virus‡
1 day	0
2 wk	0
4 "	0
9 "	0
11 "	±
13 "	±
15 "	+
18 "	+

* At various times post-LDV virus pools were collected. Serial tenfold dilutions of each pool were then incubated with goat anti-mouse sera. Normal goat sera served as the control. Each set of dilutions was injected into mice and the neutralization index (NI) was determined.

‡ 0 = negative, NI less than 0.5 log units; ± = equivocal, NI 0.5 to less than 1.0 log units; + = positive, NI over 1.0 log units.

diluted in ice cold EBMV and the virus titer was determined. The data in Table XI show that the residual infectious virus which was not neutralized by mouse anti-LDV (group C) reacted with goat anti-mouse sera (group D), whereas virus incubated with normal mouse sera (group A) failed to react with goat anti-mouse sera (group B). From these experiments it is concluded that LDV can be sensitized in vitro by incubation with anti-LDV.

Resistance of in Vivo Sensitized Virus to Neutralization by Anti-LDV.—The fact that infectious virus existed in the circulation of mice in the presence of neutralizing antibody suggested that the sensitized complex was relatively resistant to neutralization by anti-LDV. To see if this were the case, approximately $10^{8.0}$ID$_{50}$/ml of sensitized virus which was obtained from mice that had been infected for 3 to 4 months was incubated at 37°C for 1 hr with anti-LDV. The reaction mixtures were then serially diluted and the virus titer was determined. As seen in Table XII, the sensitized virus was relatively resistant to

neutralization by anti-LDV. Table XIII shows that resistant virus was not found in the circulation until the 14th wk of the infection. This corresponds

TABLE X

Effect of Anti-Mouse Sera on Progeny of Sensitized Virus

Type of virus*	Sera (goat)	Virus titer ID_{50}/ml (log 10)
Parent (sensitized)‡	Normal	3.8
	Anti-mouse	1.8
Progeny§	Normal	3.1
	Anti-mouse	3.1

* The parent virus (sensitized) and its progeny were diluted 1 in 20 and incubated with goat anti-mouse sera or normal goat sera for 1 hr at 37°C. The reaction mixtures were then serially diluted and titered.

‡ Parent (sensitized) virus was obtained from mice that had been infected with LDV for 10 months.

§ Progeny was obtained from mice 15 hr after intraperitoneal injection of 0.1 ml of a $10^{-3.0}$ dilution of the parent (sensitized) virus.

TABLE XI

*In Vitro Sensitization of Virus**

Group	Incubation I (mouse sera)	Incubation II (goat sera)	Virus titer ID_{50}/ml (log 10) Experiment No. 1	2	3
A	Normal	Normal	>4.3	4.4	4.7
B	Normal	Anti-mouse	>4.3	4.4	4.4
C	Anti-LDV‡	Normal	3.3	3.5	3.4
D	Anti-LDV‡	Anti-mouse	2.2	2.1	2.8

* Unsensitized stock virus (approximately $10^{8.0}$ ID_{50}/ml) was incubated with normal mouse sera or mouse anti-LDV at 37°C for 1 hr (incubation I). Each reaction mixture was then diluted $10^{-3.2}$. To aliquots of this latter dilution, normal goat sera or goat anti-mouse sera was added and incubated in the usual way at 37°C for 1 hr (incubation II). Incubation II was then serially diluted and the virus titer was calculated.

‡ Sera from mice that had been infected with LDV for 4 months.

closely to the time of appearance of anti-LDV and sensitized virus (Tables I and IX).

If the resistance of the virus to neutralization by anti-LDV were related to sensitization, then the unsensitized progeny should be susceptible to anti-LDV. If, on the other hand, the resistant virus represented a genetic variant, then its

progeny should also be resistant. To differentiate between these possibilities, the resistant virus (sensitized) was diluted 1 in 1000 and 0.1 ml was injected intraperitoneally into recipient mice. 15 hr later the unsensitized progeny was

TABLE XII

Resistance of in Vivo Sensitized Virus to Neutralization by Anti-LDV‡*

Reaction mixture§	Virus titer ID_{50}/ml (log 10)		
	Experiment No.		
	1	2	3
Normal sera	3.0	2.9	2.9
Anti-LDV	3.2	2.7	2.8

* Sensitized virus was obtained from mice that had been infected for 3 to 4 months.

‡ Anti-LDV was obtained from mice that had been infected for 4 months.

§ Approximately $10^{3.0}$ ID_{50}/ml of sensitized virus was incubated with anti-LDV or normal mouse sera at 37°C for 1 hr. The reaction mixtures were serially diluted and the virus titer was determined.

TABLE XIII

Time of Appearance of Resistant Virus in Vivo

Collection of virus (post-LDV)	Resistance (R) or susceptibility (S) of virus to neutralization as reflected by reduction in virus titer (log 10) following in vitro incubation with anti-LDV*
1 day	1.6 (S)
4 wk	1.4 (S)
11 "	1.3 (S)
14 "	0.0 (R)
43 "	0.3 (R)

* At various times after infection virus pools were collected and diluted 1 in 100 or 1 in 1000. Aliquots from each pool were then incubated for 1 hr at 37°C with stock anti-LDV or normal mouse sera. The reaction mixtures were serially diluted and the reduction in virus titer was calculated. A reduction in titer of less than 0.7 log units was taken as evidence of resistant virus (R).

collected (as in Table X) and incubated with anti-LDV. The data in Table XIV show that the unsensitized progeny was readily neutralized by anti-LDV.

Resistance of in Vitro Sensitized Virus to Neutralization by Anti-LDV.— The above experiments showed that virus which had been sensitized in vivo was relatively resistant to neutralization by anti-LDV. To see whether virus sensitized in vitro also was resistant to anti-LDV the following experiment was performed. Approximately $10^{6.0}ID_{50}$/ml of stock virus was sensitized in

vitro (as in Table XI) by incubation with anti-LDV. Aliquots of the reaction mixture were then removed and tested for resistance by incubation with ex-

TABLE XIV

*Susceptibility of Progeny of Resistant Virus to Neutralization by Anti-LDV**

Virus	Incubation (mouse serum)	Virus titer ID_{50}/ml (log 10)
Parent (sensitized)‡	Normal	2.9
	Anti-LDV§	2.8
Progeny (unsensitized)‖	Normal	3.6
	Anti-LDV§	2.2

* The resistant parent (sensitized) and its progeny (unsensitized) were diluted 1 in 20 and 1 in 100, respectively, and then incubated with anti-LDV at 37°C for 1 hr. The reaction mixtures were serially diluted and the virus titer determined.

‡ Parent (sensitized) virus obtained from mice that had been infected with LDV for 4 months.

§ Sera from mice that had been infected with LDV for about 4½ months.

‖ Progeny (unsensitized) was obtained from mice 15 hr after intraperitoneal injection of 0.1 ml of a 1 in 1000-dilution of parent (sensitized) virus.

TABLE XV

*Resistance of in Vitro Sensitized Virus to Further Neutralization by Anti-LDV**

Group	Incubation I‡	Incubation II‡	Virus titer ID_{50}/ml(log 10)	
			Experiment No.	
			1	2
A	Normal Sera	Normal Sera	4.8	>4.2
B	Anti-LDV§	Normal Sera	3.0	3.0
C	Anti-LDV§	Anti-LDV§	3.1	2.9

* Unsensitized stock virus (approximately $10^{6.0}$ ID_{50}/ml) was incubated in the usual way with 0.3 ml of anti-LDV or normal mouse sera in a final volume of 1.2 ml (incubation I). Aliquots of 0.3 ml were then removed and incubated with an additional 0.3 ml of anti-LDV or normal mouse sera in a final volume of 1.5 ml (incubation II). Incubation II was then serially diluted and the virus titer calculated.

‡ Incubated at 37°C for 1 hr.

§ Sera from mice that had been infected with LDV for 5 months.

cess anti-LDV. As seen in Table XV, the in vitro sensitized virus (groups B and C) was resistant to further neutralization. However, it should be emphasized that the degree of resistance does vary with the amount of antibody used to sensitize the virus, the amount of antibody employed in the neutralization reaction, and the time, temperature, and dilution of the reaction mixtures (9).

DISCUSSION

Because of the lifelong viremia, it was thought previously that mice made little if any neutralizing antibody to LDV (1–4). However, attempts to test for neutralizing antibody were always hampered by the presence of infectious virus in the chronic sera. The experiments reported herein showed that if measures were first taken to inactivate or dilute out the infectious virus in the chronic sera, neutralizing antibody could be demonstrated. These findings suggest that the detection of neutralizing antibody in other chronic viremias, such as the viral leukemias in mice, might be greatly facilitated if the infectious virus in the test sera is first inactivated or removed.

Although neutralizing antibody appeared at about 2½ months after initiation of the infection, the LDV-viremia persisted. The persistence of infectious virus in serum containing antibody suggested that some antibody molecules might be attached to the residual infectious particles (sensitized virus). The demonstration that goat anti-mouse sera or goat anti-mouse γ-globulin could inactivate mouse anti-LDV, pointed to the possibility that anti-mouse sera might also react with the LDV–anti-LDV complex. That this was the case was illustrated by the fact that infectious virus which persisted after in vivo or in vitro exposure to mouse anti-LDV was readily neutralized by anti-mouse sera or anti-mouse γ-globulin, whereas virus not previously exposed to mouse anti-LDV (unsensitized virus) was totally resistant to neutralization by anti-mouse sera. Thus, by the use of an anti-mouse sera we were able to show that mouse anti-LDV was actually attached to the virus particle and that sensitization did not result in loss of infectivity. From these findings it is suggested that the use of an anti-γ-globulin in other virus-antibody systems might (*a*) prove to be a useful method for detecting sensitized virus, (*b*) aid in demonstrating otherwise undetectable antiviral antibody, and (*c*) be useful in neutralizing the so-called "resistant fraction". Support for these suggestions comes from recent experiments in which we sensitized herpes simplex virus with an antiherpes serum made in rabbits and found that we could neutralize over 99.9% of the infectious herpes-antiherpes complex with an anti-rabbit serum made in goats (10).

The experiments described above showed that sensitized virus could be neutralized by antibody which was not directed against the virus particle itself. The mechanism of neutralization of sensitized virus requires further study, but we should like to enumerate what we consider the most likely possibilities. First, anti-mouse sera or anti-mouse γ-globulin might act by attaching to and/or forming a bridge between anti-LDV molecules which are bound to the surface of the sensitized particle so as to block critical sites (areas which must not be altered or blocked if the virus is to remain infective). Second, anti-mouse sera might form a lattice between sensitized particles. However, the neutralization of extremely low concentrations of sensitized virus (less than 100 ID_{50}/ml) by anti-mouse sera is difficult to explain on the basis of lattice formation. Third,

anti-mouse sera or anti-mouse γ-globulin might interact with the anti-LDV which is attached to the virus particle in such a way as to pull the anti-LDV off the virus and in so doing disrupt or damage the infectious particle with resulting loss of infectivity. Further studies are required to differentiate between these possibilities.

The persistence of a relatively resistant virus fraction following in vitro incubation with antiviral antibody is not unique for LDV. Similar findings have been reported with a number of other viruses including polio, western equine encephalitis, Newcastle disease, influenza, rabbitpox, Japanese B encephalitis, foot-and-mouth disease, visna, and herpes simplex (11–25). The present study with LDV, however, showed that in addition to a resistant fraction in vitro, a similar fraction exists in vivo soon after the appearance of circulating antibody. In addition, our studies showed that the resistant fraction, in contrast to the susceptible fraction, was sensitized with anti-LDV. These observations suggest that sensitization of the virus might hinder neutralization and produce the so-called resistant fraction. A somewhat similar hypothesis has been proposed to account for the resistant fraction in other virus-antibody systems (13–19, 21, 22, 25–27). Recent studies on the interaction of virus and antibody which employed density gradient centrifugation, electrophoretic mobility, and an aqueous polymer phase system provide tentative support for this hypothesis (18, 25–27). These experiments showed that the infectious virus which persisted after exposure to antiviral antibody migrated with or was found in association with the globulin fractions. Our demonstration that an anti-γ-globulin could in fact neutralize the resistant virus showed that the antiviral antibody was actually attached to the infectious particle. Although these findings pointed to viral sensitization as the most likely explanation for resistance, the existence of a genetic or phenotypic variant which was insusceptible to neutralization but not to sensitization had to be ruled out. The susceptibility of the progeny of the resistant virus to neutralization by anti-LDV (Table XIV) argues against a genetic variant. A phenotypic variant seems unlikely on the basis of preliminary experiments (discussed below) which indicate that the degree of resistance is a function of the neutralization conditions. Furthermore, to explain the resistance of LDV or that of other viruses in terms of a phenotypic variant would require additional and more complicated assumptions than are warranted on the basis of available evidence.

Finally, we should like to consider the relationship between viral neutralization, sensitization, and resistance. If one postulates that on each virus particle there are critical and noncritical sites, then attachment of antibody to critical sites would result in viral neutralization. On the other hand, attachment of antibody to noncritical sites would result in sensitization. If some of the noncritical sites are in close proximity to the critical sites, attachment of antibody to these noncritical sites might sterically hinder free antibody molecules from reaching the critical sites. Consequently these particles would be rela-

tively resistant to neutralization. The degree of resistance to neutralization would depend on the extent of the sensitization and the sites involved. Evidence for this latter contention comes from recent studies (9), which showed that a dilution of anti-LDV which was insufficient to neutralize LDV was adequate to sensitize the virus. However, the degree of sensitization in this case was inadequate to protect the virus from neutralization by undiluted anti-LDV. On the other hand, as shown in this paper, a concentration of anti-LDV which neutralized close to 99% of the virus, left a residual sensitized fraction which was relatively resistant to further neutralization. Whether the sensitization and neutralization of LDV are produced by the same or different types of antibody is not known. Lafferty (18, 27) recently found that papain-digested monovalent antibody protected influenza virus from neutralization by undigested divalent antibody. Similarly, if there are two types of anti-LDV, the attachment of "sensitizing" or "nonavid" antibody (28) to critical and/or noncritical sites might protect the virus from neutralizing antibody and produce a resistant fraction. Although it is not possible at the present time to differentiate between these alternatives, we believe that further studies on the mechanism and kinetics of virus sensitization may aid in elucidating the factors which affect the susceptibility and resistance of a virus to neutralization.

SUMMARY

If viremic sera from mice chronically infected with lactic dehydrogenase virus (LDV) were first treated with ether or ultraviolet light to inactivate the infectious virus, neutralizing antibody could be demonstrated. Significant amounts of antibody, however, were not detected until the mice had been infected for about 2½ months and its presence did not result in the elimination of the chronic viremia. Virus isolated from sera containing neutralizing antibody was found to be relatively resistant to neutralization by anti-LDV. Further studies revealed that the resistant virus existed in the form of an infectious virus–antibody complex (sensitized virus). The presence of such a complex was demonstrated by the fact that the virus fraction which persisted after in vivo or in vitro exposure to mouse anti-LDV was readily neutralized by goat anti-mouse sera or goat anti-mouse γ-globulin, whereas virus that had not been previously exposed to mouse anti-LDV was completely resistant to neutralization by goat anti-mouse sera. These findings suggest that (*a*) sensitization may play an important role in the resistance and susceptibility of a virus to neutralization by antiviral antibody, and (*b*) an anti-γ-globulin may prove useful in neutralizing the resistant fraction and in demonstrating otherwise undetectable antiviral antibody.

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