Virucidal effect of murine duodenal extracts: studies with lactate dehydrogenase-elevating virus

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Summary

Mucosal resistance to infection with lactate dehydrogenase-elevating virus (LDV) has been previously demonstrated, and the LDV system presents an important murine model for the study of mucosal barriers to viral infection. In the present study, duodenal molecules were isolated from normal mice which had potent virucidal activity, when tested against LDV as well as canine herpes, canine hepatitis, Semliki forest, and visna viruses. The virucidal activity was demonstrated to be non-immune in nature, and was present in apparently non-enzymatic protein molecules, having a molecular mass of between 10–100 kDa by membrane filtration and 10–17 kDa by gel filtration. The anti-LDV activity of these molecules was suppressed by anti-duodenum antibodies in vitro, and in vivo studies suggested a possible protective role for the anti-viral molecules. We conclude that the normal mouse duodenum contains potent virucidal molecules, which are of interest to the study of biological and molecular mechanisms of viral resistance.

Duodenal extract; Lactate dehydrogenase-elevating virus; Mouse

Introduction

Mucosal surfaces play an important role in protecting the potential host from viral infection, and thus are of significance to the pathogenesis of a variety

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of human and animal viruses. Protection at mucosal sites may be mediated by physical or chemical mechanisms, which restrict the attachment of viruses to host target cells (Braude, 1981). Viral infection at intact mucosal sites may require a large number of virions, i.e. a minimum infectious dose (MID) sufficient to overwhelm mucosal defense mechanisms (Ward and Akin, 1984). Although these defense mechanisms are incompletely understood, they may include both antibody (specific) as well as non-specific molecules which can disable viruses (Ward and Akin, 1984). The presence of non-specific anti-viral molecules has been described in human saliva (Fultz, 1986; Archibald and Cole, 1990) and bovine milk (Panon et al., 1987), and such molecules may represent protective barriers to virus infections.

Lactate dehydrogenase-elevating virus (LDV) is a murine togavirus which causes a persistent infection (Rowson and Mahy, 1985; Cafruny, 1989). The mechanism of persistence is related to infection of a permissive subpopulation of macrophages which continuously evolves in vivo, as well as inability of the immune response to clear the virus (Rowson and Mahy, 1985; Cafruny, 1989; Bradley et al., 1991). There is a powerful relative mucosal barrier to LDV infection, demonstrated by a MID of over 100,000 virions for orally administered LDV, in contrast to a MID of one virion by parenteral exposure (Cafruny and Hovinen, 1988). The nature of the mucosal barrier to LDV infection is not clear, but it consists in part of non-specific biochemical factors which appear to be prostaglandin-sensitive (Cafruny et al., 1991).

For the present study, LDV-inhibitory molecules were investigated in the non-immune mouse gastrointestinal tract. Extracts from mouse small intestine were characterized as to their anti-LDV activity, and studies on the anti-viral spectrum and chemical nature of these extracts were also carried out. The data show that the mouse duodenum contains potent anti-viral molecules, which are virucidal for a variety of unrelated viruses.

Materials and Methods

Mice

Female outbred mice of the CF1 strain were obtained at about 8 weeks of age from Sasco, Inc. (Omaha) or Amitech (Omaha). Mice were maintained on standard lab chow and tap water ad libitum.

Tissue extracts

Mice were sacrificed by cervical dislocation and the stomach and intestines removed and placed in cold phosphate-buffered saline (PBS) pH 7.4. The intact gut was rinsed well with cold PBS, and various segments as described were placed in a glass tissue grinder. After complete grinding of the tissues, tissue remnants were allowed to settle for several minutes, and the supernatant was clarified by centrifugation at 12,000 × g for 10 min. Following sterile filtration through 0.45-μm filters, protein determinations were carried out by measuring

optical density at 280 nm or by a dye-binding assay (BioRad), and total protein was standardized at about 23 mg/ml for all extracts. The extracts were stored at −80°C. In some experiments extracts of other organs were made by an identical procedure. For preparation of mucosal extracts, the duodenal mucosa was scraped free of the intestinal wall and suspended in PBS. Brush border membranes were prepared as described (Kessler et al., 1978). Small intestine T-cells were obtained by non-continuous gradient floatation as described previously (Dillon and MacDonald, 1984).

Physical and chemical treatments

Enzyme inhibition was carried out as described previously (Gaspari et al., 1988). Briefly, tissue extracts were incubated with soybean trypsin inhibitor (1 mg/ml), EDTA (1 M), phenylmethylsulfonyl-fluoride (100 mM) and sodium azide (0.2 mg/ml) for 15 min at 37°C. At the end of the incubation period, the treated extracts were compared with control untreated extracts for anti-LDV activity. IgG and IgA were absorbed from tissue extracts with appropriate affinity columns [protein A-Sepharose for IgG; an anti-mouse IgA column prepared with affinity-adsorbed anti-mouse IgA (Southern Biotechnology) conjugated to cyanogen bromide-activated Sepharose 4B for IgA]. The specificity of the adsorptions was demonstrated by Western blot analyses, which also showed that all detectable IgG or IgA were removed from crude extracts by the affinity columns.

Heat treatments were carried out by heating 0.1-ml aliquots in a block heater for 15 min. In some experiments, tissue extracts were precipitated in 50–60% ammonium sulfate for 30 min, followed by centrifugation at $10,000 \times g$ for 30 min, and the supernatant was collected. Ultracentrifugation and filtration of tissue extracts were carried out as described in the text.

Virus assays

LDV stocks of the Plagemann strain (LDV-P, Cafruny and Plagemann, 1982) were prepared from the plasma of mice at 24 h post-infection (p.i.) and titrated as described (Plagemann et al., 1963). Briefly, 10-fold serial dilutions of the virus stocks were prepared in cold PBS, and 0.1 ml of each dilution was injected intraperitoneally into indicator mice. Four days following the injections, indicator mice were bled and plasma LDH levels were determined. The titration end-point was calculated from the highest dilution which yielded the rise in plasma LDH characteristic of LDV infection. Stock virus was frozen in 0.1-ml aliquots, containing about 10^9 ID_{50}/ml. Inactivation of LDV was assessed in vitro, by comparing the concentration of infectious LDV in experimental tubes containing various test extracts with the concentration of LDV in control tubes. For most experiments, tissue extracts containing a total protein concentration of approximately 23 mg/ml were incubated in a final volume of 0.1 ml with 10^7 ID_{50} of LDV, or with other viruses as described in the text. LDV-RNA was labeled with [^{3}H]uridine and analyzed by sucrose density ultracentrifugation as described previously (Brinton-Darnell and Plagemann, 1975).

Canine kidney (MDCK-USD) cells (Gaush and Smith, 1968) cultured in Eagles MEM supplemented with 5% fetal bovine serum were used for the growth of canine herpes virus (CHV-14, ATCC RR no. 532, strain D004), infectious canine hepatitis virus (Lederle Research Reference Reagent), and vaccinia virus (obtained from Dr. C. Gaush, American Red Cross Laboratory, Bethesda, MD). The viruses were titrated by a plaque assay essentially as described previously using 3 culture plates per dilution (Gaush and Smith, 1968). Semliki forest virus (ATCC, 11th passage in mouse brain) was grown in an established deer kidney cell line cultured in D-MEM supplemented with 5% FCS. Visna virus (ATCC VR-779) was grown in sheep choroid plexus cells cultured in Medium 199 supplemented with 2% lamb serum. Confluent cells were inoculated in 6-well plates with 0.5 ml of virus, and then incubated at 37°C for 10–12 days. The cells were fixed in 10% buffered formalin, stained with 1% crystal violet, and plaques were counted in triplicate cultures.

Column chromatography

Small intestine extracts were clarified at 12,000 × g for 20 min, filtered through 0.45-μm filters, and separated on Sephadex G200 or G75 columns. The columns (102 × 2.2 cm) contained a total void volume of about 105 ml, as determined with dextran blue, and the total bed volume was about 350 ml. Small intestine extracts (70 mg total protein) or molecular weight marker were run through the columns in buffer containing 14 mM NaH_2PO_4, 36 mM Na_2HPO_4, and 0.5 M NaCl (G200), or 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 27 mM KCl, and 0.14 M NaCl (G75). Fractions (5 ml) were collected, absorbency at 280 nm determined for each fraction, and 5 or 6 consecutive fractions were serially pooled, dialyzed for 24 h against distilled H_2O, frozen at −70°C, and lyophilized. For analysis of anti-viral activity, the lyophilized column fractions were reconstituted in 0.2–0.4 ml PBS and incubated with virus stocks for 30 min at 37°C.

Immune inhibition of duodenal LDV inhibitor

A young adult New Zealand white rabbit was immunized with extracts prepared from mouse small intestine. The first injection of 5 mg of mouse duodenum was given in Freund's adjuvant and administered subcutaneously, and was followed by 4 weekly injections of 5 mg of duodenum extract given i.v. On weeks 12 and 13, the rabbit was immunized with 2.5 mg duodenum i.v. Monitoring of the anti-duodenum titer by ELISA indicated increasing levels of anti-duodenum IgG, and on week 14 the rabbit was sacrificed and serum was collected, which had an anti-duodenum IgG titer of 1:256,000 by ELISA. The antiserum also displayed broad reactivity with mouse duodenum by Western immunoblot (data not shown). This serum was used for oral administration by pipet to normal mice, prior to exposure to a limiting dose of LDV by the oral route (Cafruny and Hovinen, 1988). The mice were then evaluated 4 days later for LDV infection. This rabbit serum was also tested for anti-LDV inhibitor activity by direct in vitro mixing with LDV inhibitor. Control rabbit serum

from an unimmunized rabbit was used in these experiments, and control antibody from a rabbit immunized with an irrelevant antigen (Mycoplasma fraction D) and Freund's adjuvant was also used to demonstrate specificity.

In vitro LDV infection

Cultured mouse macrophages were infected with LDV at a multiplicity of 500–1000 as described previously (Stueckemann et al., 1982). Detection of infection was by fluorescent antibody analysis of the infected cells, which were fixed with acetone at 8 h p.i. (Cafruny et al., 1986).

Results

Inactivation of LDV by extracts of normal mouse small intestine

In initial experiments, small intestines were excised from normal CF-1 mice, washed in pH 7.4 PBS, and ground in a glass tissue grinder with 1–2 ml of PBS. The intestinal extracts were clarified by centrifugation at 12,000 × g for 10 min at 4°C, filtered through 0.45-μm filters, and stored at −80°C. Total protein content was standardized at approximately 23 mg/ml. Reaction tubes were set up which contained 90 μl of tissue extract and 10 μl of LDV (final virus concentration = 10^8 ID$_{50}$/ml). As shown in Fig. 1, extracts of mouse small intestine were found to inactivate LDV in vitro prior to injection into indicator mice (Fig. 1). The absence of a lag phase in the kinetics of inactivation indicates
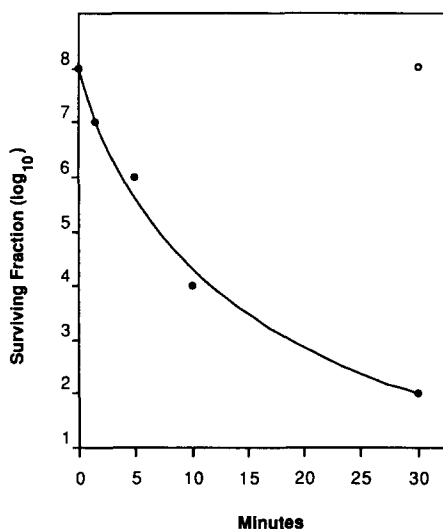

Fig. 1. Inactivation of LDV by mouse small intestine extract. LDV at a concentration of 10^8 ID$_{50}$ ml was incubated with mouse duodenum extract (total protein concentration = 23 mg/ml) at 37°C. Samples were taken from the reaction tube at the time shown and titrated for infectious LDV. Incubation of LDV in a control tube containing PBS (open circles) did not reduce the titer.

TABLE 1

Tissue distribution of LDV inhibitor

Tissue[a]	No. Expts.	Mean inhibition $(\log_{10})$[b]
Duodenum	4	2.2
Ileum	3	0.2
Jejunum	3	1.3
Stomach	4	0.0
Lung[c]	2	1.0
Kidney	2	0.0
Heart	2	0.0
Liver	2	0.0
Spleen	2	0.0

[a]Tissue extracts were prepared from normal CF1 mice as described in Materials and Methods. Total protein concentration was equalized to about 23 mg/ml.
[b]Mean inhibition of a starting virus concentration of 10^8 ID_{50}/ml is shown. LDV titers were determined using 2 or 3 mice/$\log_{10}$ dilution. Reaction tubes were sampled for titration immediately before, and after 10 min of incubation at 37°C, addition of tissue extracts.
[c]Inhibitory activity was seen in only one experiment where incubation was extended to 30 min at 37°C.

a single-hit mechanism of viral inactivation (Mandel, 1979). Incubation of virus in control tubes containing PBS or normal mouse plasma failed to result in any virus inactivation during the time period studied.

Experiments were also performed in which LDV was incubated with extracts of normal mouse duodenum, ileum, jejunum, stomach, liver, spleen, heart, lung, and kidney. As shown in Table 1, virucidal activity was concentrated in the duodenum, with lower concentrations observed in ileum, jejunum, and lung. Thus, the viral inhibitory molecules were anatomically restricted, and LDV inactivation was not simply due to the presence of comparable concentrations of mouse proteins, or other biomolecules such as enzymes, present in tissues other than duodenum. LDV inhibitor was not observed in brush boarder membranes or T-cell-enriched fractions of the duodenum when these purified fractions were tested at a protein concentration of about 15 mg/ml (data not shown), further demonstrating restricted localization of the inhibitor within cells of the duodenum.

LDV inactivation by duodenal extracts was temperature-dependent, since the rate of viral inactivation was greater at 37°C than at 22° or 4°C (data not shown). In addition, the anti-LDV effect was dependent in a linear fashion on the concentration of extract used in the reaction tube, and small intestine extracts with an A_{280} of less than about 0.2 (about 5 mg/ml by dye-binding protein assay) were rarely active against high titers of the virus (data not shown).

Size and chemical resistance of duodenal LDV inhibitor

The hypothesis that LDV inactivation by mouse duodenum extracts was due to enzymes was tested by preincubating duodenum extracts with several compounds known to inactivate a range of proteases normally present in

TABLE 2

Chemical-physical characterization of duodenal LDV inhibitor

Treatment[a]	LDV Titer ($\log_{10}$/ml)[b]	
	Pre	Post
none	8.0	4.0
enzyme inhibitors	8.0	4.0
ammonium sulfate (50% supernatant)	8.0	8.0
ammonium sulfate (60% pellet)	7.0	2.0
100 kDa filter	8.0	4.0
10 kDa filter	8.0	8.0
65,000 × g supernatant	8.0	8.0
65,000 × g pellet	8.0	4.0
mouse food	8.0	8.0

[a]LDV at a concentration of 10^8 ID_{50}/ml was incubated with duodenal extracts which were pretreated as indicated in the table and described in the text. The LDV titer was determined before (pre) and after (post) incubation at 37°C for 10 min with the untreated or treated extracts, or with an extract of mouse food. See text for methods.
[b]LDV titers shown ± 0.25 $\log_{10}$ units.

mammalian gut (Gaspari et al., 1988). Pretreatment with phenylmethylsulfonyl fluoride (PMSF), sodium azide, EDTA, and soybean trypsin inhibitor had no effect on the ability of mouse duodenal extracts to inactivate LDV, as shown in Table 2. Table 2 also shows results of experiments in which the duodenal LDV inhibitor was precipitated in 50–60% ammonium sulfate, or was pelleted by centrifugation at 65,000 × g for 150 min. The inhibitor was recovered by resuspension in PBS of 60% ammonium sulfate, as well as the ultra-centrifuged pellets. The inhibitor also passed through filters with a MW cutoff of 100,000 Daltons, but was retained on filters with a cutoff of 10,000 Daltons (Table 2). To determine that the inhibitor was not an exogenous food product, solubilized mouse food was shown to have no anti-LDV effects (Table 2). Taken together, these data show that the LDV inhibitor from mouse duodenum is a protein-like 10–100 kDa macromolecule, and suggest a possible non-enzymatic mechanism of action.

Heat stability of duodenal LDV inhibitor

Duodenal extracts were subjected to heating before testing for anti-LDV activity. Treatment at 56°C for 30 min enhanced the anti-LDV activity, since $10^8 ID_{50}$/ml of virus were reduced to $10^6 ID_{50}$/ml by untreated duodenal extract, and to $10^4 ID_{50}$/ml by heated extract (data not shown) and this effect was routinely observed in a number of experiments. At 100°C, anti-LDV activity was retained to the same extent as in unheated preparations, but at 120°C the anti-LDV activity was destroyed (data not shown). These results show that duodenal anti-LDV activity was not due to a complement-mediated reaction, and further support the non-enzymatic nature of the anti-LDV activity.

Non-immune nature of duodenal LDV inhibitor

Mouse duodenal extracts were absorbed with either protein A-Sepharose or an immunoadsorbant prepared from activated Sepharose and affinity-purified goat anti-mouse IgA. These treatments removed detectable IgG and IgA, respectively, from the mouse extract, as determined by Western analyses (data not shown). In both experiments, the absorbed extracts retained their full anti-LDV activity (>99% inactivation, data not shown), showing that the activity was not due to the presence of IgG or IgA antibodies.

Gel filtration of intestinal LDV inhibitor

When small intestine extracts were fractioned on Sephadex G200, the fractions corresponding to molecular mass > 100 kDa contained about 50% of the total protein eluted, with smaller amounts of protein distributed throughout the remainder of the column fractions (data not shown). Table 3 shows results of 3 independent experiments, in which different small intestine extracts were fractioned on Sephadex G200 and tested for anti-LDV activity. Inhibition of >99% of viral infectivity was seen only with fractions corresponding to molecular weight of about 10–17 kDa, as estimated from standard curves prepared with carbonic anhydrase (29 kDa) and alcohol dehydrogenase (150 kDa). Other fractions containing more total protein, as well as a sham-prepared fraction consisting of eluted PBS, displayed no anti-LDV activity. These results show that a significant purification of the small intestine viral inhibitor is possible, since Sephadex fractions containing anti-viral activity had total protein concentrations ranging between 0.2 and 0.4 mg/ml.

Fractionation of small intestine extracts on Sephadex G75 resulted in essentially identical data as were obtained by G200 fractionation. As shown in Table 3, anti-LDV activity was contained only in the G75 column fractions corresponding to molecular weight of approximately 10 kDa. When the LDV-inhibitory G75 column fraction was tested against Semliki forest virus (pre-treated titer $= 5 \times 10^7$ PFU/ml), there was approximately 94% inactivation (post-treated titer $= 3 \times 10^6$ PFU/ml).

TABLE 3

Gel filtration of mouse small intestine LDV inhibitor

Expt.	LDV Titer[a]		% Inactivation
	Pre-incubation	Post-incubation	
G200-1	10^7	10^5	$\geqslant 99$
2	10^7	10^5	$\geqslant 99$
3	10^7	$< 10^4$	$\geqslant 99.9$
G75-1	10^8	$< 10^5$	$\geqslant 99.9$
2	10^7	10^6	$\geqslant 99$

[a]LDV was incubated with 10–17 kDa G200 or G75 fractions of small intestine extracts for 30 min at 37°C, and the pre- and post-incubation titers were determined. Other column fractions were similarly assayed with no detectable anti-LDV activity (data not shown). LDV titers shown ± 0.25 $\log_{10}$ units.

Structural analysis of duodenal extract-treated LDV

LDV was labeled with [³H]uridine during growth in cultured macrophages (Brinton-Darnell and Plagemann, 1975). Fig. 2 shows the sucrose density gradient profile of labeled intact virions, and also the results of analyses of labeled virions which were treated with duodenal extract prior to centrifugation on 0.5–1.5 M sucrose density gradients. Untreated intact virions migrated within sucrose gradients to a single major peak, representing a density of about 1.14 g/cc (Brinton-Darnell and Plagemann, 1975). Pretreatment of labeled intact virions with 30 mg/ml of heated (56°C for 30 min) mouse duodenal extract for 10 min at 37°C had little or no effect on the sucrose density gradient profile (peaks within 0.5 ml in the representative experiment shown in Fig. 2), suggesting that LDV inactivation by the mouse duodenal inhibitor was not due to gross disruption of the virion. In contrast, during incubation of intact labeled virions with non-heated duodenal extract there was extensive disruption of the virion, as shown by loss of the [³H]uridine-labeled peak. These results are explained by the sensitivity of tissue-culture-derived LDV to enzymes such as trypsin (Kowalchyk and Plagemann, 1985; Broen and Cafruny, unpublished data), in contrast to blood plasma-derived virus, which is protected from the action of trypsin and probably other factors (Crispens, 1965; Broen and Cafruny, unpublished data). Thus, heating of duodenal extract eliminates extraneous factors which disrupt tissue-culture-derived virus, but preserves anti-LDV activity and allows its analysis in the absence of other factors which may disrupt the virion.
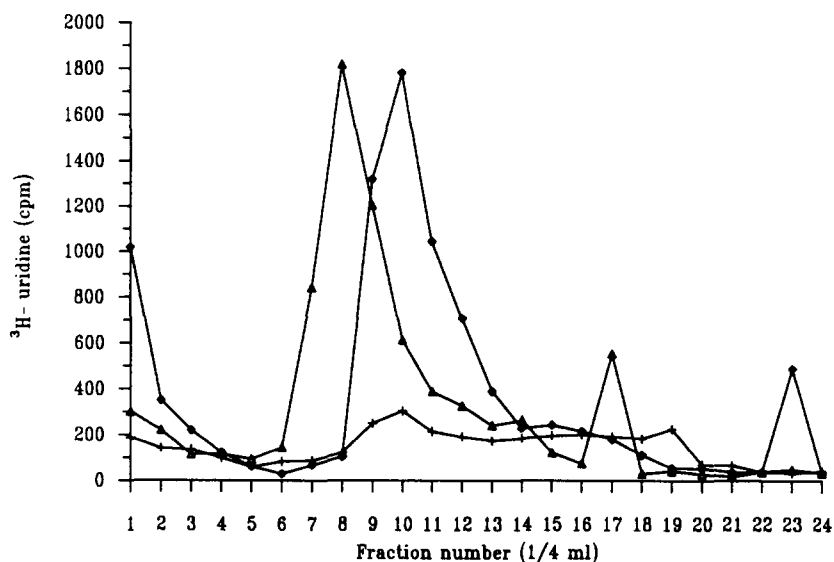

Fig. 2. Sucrose density gradient analysis of LDV. LDV labeled with [³H]uridine was fractionated on 0.5–1.5 M sucrose density gradients (Brinton-Darnell and Plagemann, 1975). Standard labeled virus (▲) displayed a peak at about 1.14 g/cc (Brinton-Darnell and Plagemann, 1975). This virus was also analyzed after 10 min incubation with duodenal extract (+) or duodenal extract heated at 56°C for 30 min (♦).

TABLE 4

Effect of rabbit anti-mouse duodenum antibody on LDV inhibitor

Treatment	n	% positive
Expt. A. In vivo titration		
Normal rabbit IgG	44	27
Rabbit anti-duodenum	44	48, $p < 0.05$

Treatment	% infection
Expt. B. In vitro infection	
LDV only	17
Duodenal inhibitor	0
Inhibitor + Anti-duodenum Ab	21
Anti-duodenum Ab only	18
LDV only	20
Duodenal inhibitor	0
Inhibitor + control Ab	0
Control Ab only	21

For the in vivo experiment (A), mice were dosed at times 25 and 10 min pre-LDV-exposure with 25 μl rabbit anti-mouse duodenum or normal rabbit serum. % positive (Expt. A) refers to the % of mice becoming LDV infected after oral LDV challenge. n = number of mice (cumulative) from 5 consecutive experiments. In Expt. B, the LDV inhibitor was incubated with rabbit anti-mouse duodenum for 30 min at 37°C, prior to 10 min of incubation with LDV at 37°C and inoculation of mouse macrophage cultures (Gaspari et al., 1988; Cafruny and Plagemann, 1982). The anti-duodenum antibody alone had no effect on the replication of LDV in mouse macrophage cultures. A separate control experiment is shown in which control Ab from a rabbit immunized with an irrelevant Ag (Mycoplasma fraction D) and Freund's adjuvant failed to inactivate the duodenal inhibitor. Representative data are shown for two independent experiments (Expt. B) indicating the % of cultured macrophages becoming LDV infected during in vitro infection.

Demonstration of anti-inhibitor antibodies

Rabbit anti-duodenum antibody was prepared and studied for activity against the LDV inhibitor. When mice were given two oral doses of the rabbit anti-duodenum antiserum prior to oral challenge with a limiting dose of LDV, there was a significant increase in the rate of LDV infection, relative to mice which were given control normal rabbit serum (Table 4A). Anti-inhibitor activity of the rabbit antiserum was also demonstrated in vitro, since incubation of LDV with the inhibitor for 10 min prior to infection of cultured mouse macrophages resulted in abrogation of the virus infection (a representative experiment is show in Table 4B), and this effect was reversed in the presence of the anti-duodenum antibody. Control experiments employing normal rabbit serum and rabbit antibodies to an irrelevant antigen (Table 4B) demonstrate the immune specificity of the anti-duodenum antibody.

Anti-viral spectrum of mouse duodenum extracts

Infectious canine hepatitis virus, canine herpes virus, vaccinia virus, Semliki forest virus or visna virus were incubated with normal mouse duodenal extracts for 10–30 min at 37°C, and with appropriate control preparations, before infection of indicator cells and determination of the virus titers by plaque assay.

TABLE 5

Inactivation of viruses other than LDV by mouse duodenal extracts

Virus	No. expts.	PFU		% inactivation
		Control	Experimental	
Canine herpes	3	1.46×10^6	0.43×10^6	70.5
Canine hepatitis	2	3.93×10^6	1.04×10^6	74.2
Vaccinia	7	2.90×10^5	3.40×10^5	0.0
Visna	1	5.80×10^4	1.00×10^3	98.0
Semliki forest	1	6.14×10^5	$<1.00 \times 10^3$	>99.0

The herpes, hepatitis, and vaccinia viruses were plaqued on MDCK cells as described in Materials and Methods. Visna was grown on sheep choroid plexus cells. The visna data shown are for a virus dilution (1:50,000) which gave the maximum quantifiable number of plaques. Similar data were obtained with lower and higher virus dilutions, which yielded between 30 and over 120 control plaques. Semliki forest virus was plaqued on deer kidney cells.

As shown in Table 5, the hepatitis, herpes, Semliki forest, and visna viruses were all susceptible to the virucidal effect of the mouse duodenal extracts while vaccinia virus was resistant. In contrast to the case with LDV, extracts from other mouse tissues showed varying degrees of viral inhibition against these viruses (data not shown). For example stomach extracts were found to inhibit canine herpes, hepatitis, and vaccinia viruses, but in the case of stomach, heating at 56°C for 20 min abrogated inhibitory activity against canine hepatitis virus. Visna virus was inhibited by mouse stomach extract to about the same extent as with duodenum extract; we have not assessed the effect of heating the stomach extract in the visna system. Pretreatment of the tissue culture cells with duodenum or small intestine extracts had no effect on virus replication, showing that the anti-viral effects were not due to inhibition of cellular metabolism or cytotoxic activity.

Discussion

Our results are significant in describing apparently novel anti-viral molecules, whose action is virucidal and not limited to a particular virus or class of viruses. These protein-like molecules are non-immune in nature, since their stability at 56–100°C eliminates the possibility of a known complement-mediated pathway, and since absorption of essentially all IgG and IgA from active crude extracts failed to diminish activity. Thus, naturally-occurring antibodies or immune factors do not play a role in duodenal anti-LDV activity. This is also the first report of a non-antibody physiological inhibitor of LDV, which is normally resistant to inactivation by whole mouse plasma as well as serum from other species.

The insensitivity of the LDV inhibitor to enzyme-deactivating agents, along with stability at 100°C, do not support an enzyme-mediated mechanism. LDV present in plasma from acutely-infected mice (stock LDV) has previously been

shown to be protease-insensitive (Crispens, 1965), although gradient-purified LDV may be inactivated by high concentrations of trypsin (Kowalchyk and Plagemann, 1985; Broen and Cafruny, unpublished data). Plasma apparently protects LDV from trypsin and possibly other enzymes, so the effects we have observed do not appear to be due to enzymatic degradation of the virus.

Our gel filtration experiments suggest that the anti-LDV activity of mouse small intestine extracts is restricted to molecules with a molecular mass of approximately 10–17 kDa. Using this estimate, a molar coefficient of anti-LDV activity of between 2.3–4.0×10^{-5} M may be calculated. These figures must be viewed as estimates only, since the molecule(s) responsible for virus inactivation have not yet been clearly identified, and they may potentially undergo some degree of dissociation or alteration during gel filtration. The gel filtration resulted in about a 175-fold purification of anti-LDV activity, which should be significant to further attempts to characterize the anti-viral activity. Inhibition of Semliki forest virus occurred with the same purified molecules which had activity against LDV, suggesting a possible dual effect of these purified molecules. However, it appears likely that other factors present in intestine or stomach extracts may be responsible for inactivation of canine herpes and vaccinia viruses, since heated stomach extracts also inactivated these viruses. This is consistent with the presence of a diverse range of potential anti-viral molecules at these sites.

It is important to note that extracts from a variety of other mouse tissues, as well as normal mouse plasma, are non-virucidal to LDV. Thus, the duodenal anti-LDV activity is not an artifact of widely-distributed tissue enzymes, binding proteins, or other components. Viral inactivation by duodenum extract molecules is clearly due to a direct effect on the susceptible viruses, since viral inactivation occurred rapidly in vitro. Furthermore, pretreatment of the cells used for the virus plaque assays failed to inhibit viral replication after exposure to a normal inoculum, as might be expected for an interferon-like mechanism.

The LDV inactivation kinetics (Fig. 1) indicate a single-hit mechanism of viral inactivation, whereby a single molecule of inhibitor binding to the virus is sufficient to disable it (Mandel, 1979). This is of interest, since mouse antibodies which are produced during LDV infection neutralize the virus by multi-hit kinetics (Cafruny and Plagemann, 1982; Cafruny et al., 1986). However, rabbit IgG has been produced to LDV which neutralized the virus by single-hit kinetics (Cafruny and Plagemann, 1982), suggesting that the interaction of duodenal inhibitor with LDV could be via the same molecular mechanism as for neutralizing rabbit antibody. LDV is an enveloped RNA virus which has three known structural proteins (Brinton-Darnell and Plagemann, 1975). The membrane-associated glycoprotein, VP-3, is the target for rabbit neutralizing antibody (Cafruny et al., 1986) as well as for recently described neutralizing mouse monoclonal antibodies (Harty and Plagemann, 1988), and provides the most likely receptor-binding molecule necessary for interaction with susceptible macrophages (Cafruny, 1989; Kowalchyk and Plagemann, 1985). LDV inactivation by duodenal molecules could be due to

analogous binding to VP-3 which disrupts the virus structure, or alternatively could result from a membrane alteration independent of VP-3 binding. Since heat-treated duodenum extracts failed to markedly alter the virion density, it is apparent that the duodenal inhibitor acts without disrupting the entire virion. It is likely that the disruption of the virion which was observed with non-heat-treated duodenum extracts was due to the contribution of additional heat-sensitive factors, including enzymes. No common inhibitor pathway can be deduced from our data, since the 5 viruses which were inhibited by duodenal molecules represent diverse viral structures and genomes.

The results of our immune studies suggest that the duodenal LDV inhibitor may play a role in physiological defense against LDV infection, since mice fed antibody to the inhibitor had a significantly increased rate of LDV infection during a subsequent oral exposure to LDV. LDV infection in vitro was also susceptible to the duodenal inhibitor, the effects of which were destroyed by incubation with anti-duodenum antibodies, providing confirmation of the immune destruction of the inhibitor molecules. The presence of natural viral inhibitors in duodenum, or other mucosal sites, would have adaptive advantage as a means to inhibit viruses at these exposed sites, and might be significant to the study of anti-viral mechanisms. Studies are in progress to further characterize viral inhibition by natural duodenal molecules.

Acknowledgements

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