Identification of a Canine Adenovirus (Infectious Canine Hepatitis Virus) Inhibitor in Dog Liver Extracts as Arginase

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Extracts of canine liver inhibited growth of infectious canine hepatitis (ICH) virus, a canine adenovirus. Purified extracts from mammalian, but not avian, liver tissue contained the inhibitor, and evidence is presented that the inhibitory factor is the enzyme arginase (arginine ureohydrolase). This study further emphasized the need for arginine in adenovirus growth and may explain some of the difficulties in isolating small amounts of ICH virus from suspensions of liver.

During studies on pathogenesis of infectious canine hepatitis (ICH) virus in this laboratory over a period of several years, two unexplained observations have been made. (i) The degree of illness and mortality rates of dogs inoculated with suspensions of infective liver generally were lower and variability in clinical response appeared greater than those of susceptible dogs inoculated by the same routes with comparable doses of virulent ICH virus propagated in dog kidney cell (DKC) cultures. (ii) When titrations of liver tissue suspensions (usually 10 to 20%v) taken from dogs at various stages of illness were performed, cytopathic effects (CPE) in DKC cultures generally occurred earlier, and were more pronounced, at dilutions greater than 1:10, especially when amounts of virus in this organ were low. These observations were given little attention until recently, when Lee (13) considered possible reasons for failures to isolate ICH virus from liver tissue samples obtained from guinea pigs after parenteral inoculation with large viral doses. This animal has been reported to be susceptible to this virus (24). The possibility of a viral inhibitor in guinea pig tissues then was considered, and a search for inhibitory activity in triturated guinea pig liver revealed a heat-labile proteinaceous substance that reduced ICH viral CPE in DKC cultures greater than 1,000-fold (13). Viral growth was inhibited, but to a somewhat lesser extent. Inhibitory activity also was found in homogenate preparations and in ammonium sulfate extracts of normal liver but not from other organs obtained from various animals (dog, cat, rabbit, pig). Although the mode of action had not been determined, it was found that the viral inhibitory factor did not act extracellularly, for viral neutralization and interference with absorption to DKC could not be demonstrated. The liver inhibitor was found active against a variety of other deoxyribonucleic acid (DNA) viruses, but it had very little effect on growth or CPE produced by selected ribonucleic acid viruses.

In further studies on the mode of viral inhibition by liver extracts (LE) prepared from normal dogs, it occurred to us that arginine might be involved. This enzyme is found almost exclusively in the liver of mammals, and its substrate, L-arginine, has been demonstrated as an essential amino acid for replication of several DNA viruses, including members of the adenovirus (8, 20), herpesvirus (3, 11), and papovavirus (10, 23) groups. A reovirus (14) also has been reported to require arginine for synthesis of infectious virus. As early as 1963, adenovirus replication was shown to have an absolute requirement for arginine; without it there was no net viral synthesis (20). This important observation emphasized the necessity for utilizing cell cultures for adenovirus growth that are free from arginine-utilizing microorganisms. Studies on the mechanism of arginine dependence for adenovirus replication have been reported recently (8).

In this report, data are presented that indicate arginase as the principal canine adenovirus inhibitor found in extracts from liver tissue.

MATERIALS AND METHODS

Cells. Tube cultures of DK cells were prepared according to standard procedures. Inoculation medium was Earle saline solution that contained 5% heated...
lamb serum, 0.5% lactalbumin hydrolysate, penicillin, streptomycin, and amphotericin B.

**Virus.** Cornell I strain ICH virus was used in all experiments. Stock virus used had been passaged 59 to 61 times in primary Dkc cultures. Repeated tests for mycoplasmal contamination of virus stock were negative.

**Preparation of LE.** Extracts from canine, bovine, chicken, and pheasant liver were prepared in three ways. (i)Crude extracts of pieces of liver that had been washed in several changes of cold isosmotic KCl (0.9%) were homogenized as a 20 or 25% suspension in a blender (Omnimixer, Sorvall). Homogenates were clarified by low-speed centrifugation (2,000 rev/min, International PR2 centrifuge) for 15 min, and the supernatant fluids then were collected and centrifuged for 30 min at 28,000 rev/min in the no. 30 rotor of a Model L ultracentrifuge (Beckman). Such extracts were termed crude LE. (ii)Crude LE was further fractionated by treatment with saturated ammonium sulfate solution to attain final saturation of 65%. The precipitate material that formed was stirred for 1 hr and, after centrifugation, was dissolved in approximately 0.25% of the original volume in distilled water. The dissolved precipitate then was dialyzed against 0.15 M phosphate-buffered saline (PBS), pH 7.2, or 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (Sigma 121), pH 7.0, until free of ammonium ions as determined with Nessler's reagent. (iii)Canine liver homogenate preparations (50%) were extracted repeatedly with cold acetone and an acetone–dried liver powder was made. This material was fractionated in six steps exactly as described by Bach et al. for purification of arginase to the precrystallization stage (fraction VI). Tests for viral inhibitory activity in concert with tests for arginase activity, noted below, were carried out at each stage of purification. Fraction VI was used for characterization studies.

**Virus titrations.** Titrations for viral infectivity were done in tube cultures by standard procedures, employing 10-fold virus dilutions and inoculums of 0.1 ml per tube. Two or three tubes were inoculated per dilution. Inoculated cultures were observed daily, and end points were expressed as median tissue culture infective dose (TCID₅₀) per 0.1 ml inoculum. End point calculations were done by the Kärber method.

**Analytical methods.** Arginase was assayed according to the method described in the *Worthington Enzyme Manual* (Worthington Biochemical Corp., Freehold, N.J., 1972), using as standard enzyme bovine liver arginase (arginine ureohydrolase, Worthington) that contained approximately 20 units/mg. Prior to assay, LE fractions were dialyzed against 0.1 M Tris-hydrochloride buffer, pH 7.0, to avoid precipitate formation with the manganese maleate buffer employed in the reaction system. Protein determination was by the biuret method, or by measurement with the Folin phenol reagent (15). Acrylamide gel electrophoresis was done by the method described by Davis and Ornstein (6), using 8-cm columns of 7.5% polyacrylamide gel (Eastman reagents) and 0.2-ml amounts of test sample material containing approximately 0.1 mg of protein dissolved in 20% sucrose. Electrophoresis was carried out at 5 mA per gel column until the tracking dye (bromophenol blue) had reached the end of the column, usually after 2.5 or 3 hr. Gel columns were removed from the glass tubing and stained with 0.05% Coomassie brilliant blue (R250) in 10% trichloroacetic acid for 3 hr, followed by repeated washes with 7% acetic acid until clarified. In some instances, slices of unstained gel that corresponded to replicate samples that had been fixed and stained were eluted into 0.1 M Tris-hydrochloride buffer, pH 7.0, and tested for arginase and ICH virus inhibitory activities. Sedimentation behavior of LE fraction VI was examined by the sucrose gradient centrifugation technique described by Martin and Ames (16). By this method, utilizing two characterized enzymes as markers (alkaline phosphatase and beef liver catalase), the sedimentation coefficients of these enzymes could be compared. Their molecular weights then could be roughly estimated. Linear sucrose (5 to 20%) gradients in PBS were prepared in 5-ml Lusteroid tubes and stored at 4 C for 4 to 5 hr before use. Samples (0.2 ml per gradient) of an enzyme mixture in PBS were layered on each gradient. Each mixture contained (per 0.2 ml) 0.1 mg of alkaline phosphatase (Worthington), 0.4 mg of dog liver fraction VI (arginase activity), and 0.1 mg of beef liver catalase (Sigma, St. Louis, Mo.). The SW39 rotor (Beckman, Palo Alto, Calif) was run at 37,500 rev/min for 1 1 hr at approximately 5 C. At the end of the runs, 10-drop fractions were collected, using a device similar to that described by Martin and Ames (16). Fractions were made up to 0.5-ml volumes with 0.01 M Tris-hydrochloride buffer, pH 7.0, and stored at −20 C until analyzed for enzyme activities and ICH viral inhibition.

**RESULTS**

**Viral inhibition by LE preparations.** Results of tests for viral inhibition by crude LE from various species are presented in Table 1. Both crude LE and ammonium sulfate precipitated (ASP) fractions from canine and bovine tissues inhibited ICH virus. However, similar materials prepared from livers of a pheasant and a chicken failed to affect ICH viral growth in Dkc cultures. Viral growth studies utilizing LE fraction IV obtained during the purification of arginase from dog liver indicated that viral replication indeed was inhibited and that the effect of LE preparations was not merely inhibition of viral CPE. A typical growth experiment where the initial ICH virus inoculum was 10⁴.₅ TCID₅₀ and 0.1 ml of a 1:10 dilution of LE was added per culture is shown in Fig. 1. The LE fraction used (fraction IV, 2.5 mg of protein/ml) had been shown in preliminary tests to inhibit growth of 500 TCID₅₀ ICH virus at a dilution of 1:128 and arginase activity (µmoles of urea per mg of protein per 30 min) was 46,700.

**Relationship between LE-inhibitor and arginase.** In Table 2, ICH viral inhibitory titers are shown in relation to arginase activity. The viral inhibitory activity was not related to the initial protein concentration of LE preparations, but it increased...
markedly with increased arginase specific activity. Those fractions that contained the enzyme always inhibited viral growth. In contrast, those fractions lacking arginase activity failed to inhibit ICH viral multiplication. Of significance was the finding that liver fractions from fowl (pheasant, chicken) neither inhibited ICH virus, nor did they have detectable arginase activity. It is known that avian species (uric acid excretors) have negligible arginase in their tissues; what little arginase occurs is present in the kidney (7).

Further evidence for arginase as the ICH viral inhibitor was obtained with ASP fractions, as well as with the more highly purified dog LE (fraction VI) subjected to rate-zonal sucrose (5 to 20%) density-gradient centrifugation. Figure 2 compares arginase activity and presence or absence of ICH viral inhibitor of 1-ml fractions collected after centrifugation of 1.0 mg of protein (fraction VI) for 34 hr at 24,000 rev/min in the SW25 rotor.

TABLE 1. Inhibition of infectious virus hepatitis (ICH) virus by liver extract (LE) preparations

<table>
<thead>
<tr>
<th>Donor animal</th>
<th>Age</th>
<th>LE preparation</th>
<th>Protein (mg/ml)</th>
<th>ICH virus titer</th>
<th>Log_{10} virus inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>12 wk</td>
<td>20% crude ASP</td>
<td>260</td>
<td>6.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Dog</td>
<td>1 hr</td>
<td>ASP</td>
<td>24</td>
<td>6.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Dog</td>
<td>1 day</td>
<td>ASP</td>
<td>13</td>
<td>5.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Calf</td>
<td>5 mo</td>
<td>20% crude ASP</td>
<td>300</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Pheasant</td>
<td>poult</td>
<td>25% crude ASP</td>
<td>128</td>
<td>5.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Chicken</td>
<td>4 mo</td>
<td>25% crude ASP</td>
<td>700</td>
<td>6.2</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* LE added in 0.1-ml amounts to dog kidney cell cultures 2 hr before virus titrations were done.
* ASP means 65%-saturated ammonium sulfate precipitate material, dialyzed against phosphate-buffered saline, pH 7.2 to 7.4, until free of ammonium.

TABLE 2. Infectious canine hepatitis viral-inhibitory and arginase activities of various liver extract (LE) preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein (mg/ml)</th>
<th>Viral inhibitory titer (^a)</th>
<th>Arginase (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude LE (dog)</td>
<td>260</td>
<td>1:16</td>
<td>630</td>
</tr>
<tr>
<td>ASP (^c) (dog)</td>
<td>24</td>
<td>1:40</td>
<td>580</td>
</tr>
<tr>
<td>ASP (neonatal dog)</td>
<td>13</td>
<td>1:16</td>
<td>420</td>
</tr>
<tr>
<td>LE (fraction VI, dog)</td>
<td>0.7</td>
<td>1:400</td>
<td>73,500</td>
</tr>
<tr>
<td>Crude LE (chicken)</td>
<td>700</td>
<td>&lt;1:2</td>
<td>0</td>
</tr>
<tr>
<td>ASP (chicken)</td>
<td>300</td>
<td>&lt;1:2</td>
<td>0</td>
</tr>
<tr>
<td>Crude LE (phea- sant)</td>
<td>470</td>
<td>&lt;1:2</td>
<td>0</td>
</tr>
<tr>
<td>ASP (pheasant)</td>
<td>128</td>
<td>&lt;1:2</td>
<td>0</td>
</tr>
</tbody>
</table>

* Titer indicates dilution of LE (0.1-ml inoculum) that caused 90% inhibition of 300 to 500 median tissue culture infective dose of infectious canine hepatitis virus.
* Arginase activity expressed as umoles of urea liberated per mg of protein per 30 min.
* ASP means precipitate fraction after ammonium sulfate (65% saturation) treatment of crude LE. Precipitate was dialyzed against phosphate-buffered saline until free of ammonium.
Again, viral inhibitory material was found only in those fractions that had arginase activity.

**Effect of arginase on ICH viral growth.** From an initial dilution of 100 μg/ml, beef liver arginase was diluted in twofold steps in cell culture medium and added (2-ml samples) to a series of DKC cultures. After 2 hr at 36°C, virus titrations were done in arginase-treated and in untreated DKC cultures. Even under these suboptimal conditions for arginase action, viral inhibitory activity was found (Table 3). For optimal arginase activity, manganese and pH 9.2 is required. In this study, no attempt was made to employ arginine-depleted media, or media supplemented with ingredients that enhance the activity of this enzyme. The optimal pH for arginase is unsatisfactory for maintenance of cell cultures.

**Reversibility of viral inhibition by arginine.** Three sets of DKC cultures were treated as follows. Set I (virus control) was inoculated with 10^4.5 TCID_{50} ICH virus. Set II (inhibitor control) was treated with 0.1 ml of a 1:10 dilution of dog LE (fraction VI) plus virus. Set III was the same as Set II, but each culture was supplemented with 10 μg of arginine-HCl adjusted with 0.1 N NaOH to pH 7.0. At intervals following virus inoculation, duplicate cultures were frozen and thawed three times and then titrated for viral infectivity. Results (Fig. 3) indicate the reversibility of the LE viral inhibitor by arginine.

**Characterization of the canine liver ICH viral inhibitor.** The viral inhibitor was precipitated by 60 to 65%, but not by 50% saturated ammonium sulfate. Full inhibitory activity also was retained by precipitation with 37.5% cold acetone. It withstood heating for 5 min at 56°C, but was inactivated by boiling for 1 min or by heating for 5 min at 70°C. Polyacrylamide gel electrophoresis of fraction VI revealed a principal, heavy band that corresponded to a similar one obtained when approximately the same quantity of beef liver arginase was subjected to electrophoresis (Fig. 4). In both preparations there were two additional very faint bands that did not correspond. Although it was possible to elute a portion of the original arginase activity from corresponding areas of a pair of unstained gel columns, viral inhibitory activity could not be recovered in detectable amounts.

By determining the relative sedimentation rates of dog LE (fraction VI) ultracentrifuged together with beef liver catalase (molecular weight 225,000) and calf intestine alkaline phosphatase (molecular weight approximately 100,000), the S_{20,w} could be roughly estimated. Replicate trials gave similar results (Fig. 5), indicating, by this method, that the molecular weight of the arginase viral inhibitor was approximately 120,000 with an S_{20,w} value of 5.3.

![Arginase and ICH viral inhibitory activities of canine liver extract preparations after sucrose gradient (5 to 20%) centrifugation.](attachment:image)

**Fig. 2. Arginase and ICH viral inhibitory activities of canine liver extract preparations after sucrose gradient (5 to 20%) centrifugation.**

![Reversion of canine LE (fraction VI) inhibition of ICH virus by arginine hydrochloride.](attachment:image)

**Fig. 3. Reversion of canine LE (fraction VI) inhibition of ICH virus by arginine hydrochloride.**

### Table 3. Effect of arginase on infectious canine hepatitis virus titers

<table>
<thead>
<tr>
<th>Arginase (μg) per DKC culture</th>
<th>Arginase effect</th>
<th>Arginase effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td></td>
<td>Virus titer</td>
<td>Titer change (log_{10})</td>
</tr>
<tr>
<td>None</td>
<td>5.5</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>5.5</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>4.1</td>
<td>-1.4</td>
</tr>
<tr>
<td>50</td>
<td>4.1</td>
<td>-1.4</td>
</tr>
<tr>
<td>100</td>
<td>3.5</td>
<td>-2.0</td>
</tr>
</tbody>
</table>

* Beef liver arginase (Worthington, Freehold, N.J.). Activity approximately 20 units/mg of protein.
* DKC, Dog kidney cell.
Fraction IV; graph liver beef 0.1-mg of virus, active reproduced from dissociable top (cathode) have been found inactivated virus, of pounds acts virus of extracellularly, most viral substances have been studied and rabbit, not (5, 352 4). FIG. Polyacrylamide gel electrophoresis patterns of 0.1-mg samples of: (1) partially purified canine LE, fraction IV; (2) purified canine LE, fraction VI; (3) beef liver arginase (Worthington). Electrophoresis from top (cathode) to bottom (anode) in 7.5% gel. Photograph is print of Coomassie brilliant blue-stained gel reproduced from color transparency.

**DISCUSSION**

A great variety of substances and conditions have been described that are capable of interfering with viral growth in laboratory hosts. Of the various nonspecific (nonimmunoglobulin) inhibitors reported active against viruses, excluding interferon, sulfated polysaccharides (heparin, substances present in commercial agar) have been most extensively studied (22). This class of compounds acts extracellularly, preventing absorption of virus to cells. Similarly, nonspecific, heat-stable inhibitors of various adenoviruses, including ICH virus, have been found in normal bovine, horse, rabbit, and swine sera. These inhibitors were found in the globulin fraction of serum, but were not specific antibody, although they formed nondissociable complexes with various adenoviruses (5, 9). In the present study, there was no evidence that virus was inactivated extracellularly. The proteinaceous inhibitory substance from liver reported active against certain DNA viruses, including ICH and canine herpesvirus, was found in extracts from liver of various normal mammals and acted intracellularly where it was postulated that it interfered with viral synthesis in an unknown manner (13).

Data introduced in the present study extend the observations of Lee (13) and indicate that the principal inhibitor of ICH viral growth found in liver extracts is arginase. Although definitive studies of the nutritive requirements for adenovirus synthesis are not available, certain amino acids such as arginine (8, 23) and methionine (18) have been shown essential for viral growth. Adenoviruses contain an internal nucleoprotein component which is approximately 23 moles percent arginine (12, 21), and it has been suggested that this amino acid may be an important factor in limiting synthesis of members of this viral group, as well as others. Recent studies by Everitt et al. (8) have confirmed the necessity of arginine for adenovirus type 2 synthesis. They showed that both cell and viral synthesis was severely impaired by complete arginine deprivation, which was reversible by addition of arginine. It was hypothesized that arginine deficiency in adenovirus infection interfered with synthesis of host- or virus-derived proteins made early in the infectious cycle. In the studies reported here, the mechanism of inhibition of ICH viral formation by liver ex-
trants was not investigated, for experiments were
done under conditions considered adequate
for cell growth, but not optimal for arginase
activity. Nevertheless, there was strong correlation
between the activity of this enzyme and the ICH
viral inhibitor. Of significance was the absence
of both inhibitor and arginase activities from
extracts of liver from avian species. Fowl liver
contains less than 0.01% arginase activity than
that of mouse liver (7). In most mammals, this
enzyme is found essentially in liver tissue only,
with very small amounts (less than 0.5%) in
the kidney and testis (7). Few other enzymes
that may be of significance as regards participa-
tion in inhibiting viral synthesis are distributed
in this manner. Lee (13) did not detect inhibitory
activity in extracts of various tissues, other than
the liver. The methods employed for purification
of the LE inhibitor, i.e., ASP and acetone ex-
traction, probably would eliminate nonspecific
lipid inhibitors, such as that isolated from brain
by Burnstein et al. (4) and found active against
measles virus. However, we did not test for
presence of lipids in LE preparations.

Ackenhusen (1) reported on an inhibitor of
viral and cellular DNA synthesis obtained from
an extract of hamster liver. The inhibitor, which
interfered with synthesis of Celo virus (an avian
adenovirus), had an estimated molecular weight
of 31,000. It appears that the hamster LE in-
hibitor differs from the one reported in this
study, since the molecular weight of the inhibitor
in canine liver was approximately 120,000. This
molecular weight estimation could vary as much
as 25%, however, for variances of 10 to 25% may
occur, depending on the enzyme standards
used (16). In any case, the dog LE that had viral
inhibitory and arginase activities had a molecular
weight greater than 100,000 and less than 150,000.
The molecular weight of beef liver arginase has
been estimated as 138,000 and the enzyme exists
in multimolecular forms (7).

It would be of interest to investigate arginase
activity of extracts prepared from cell cultures
derived from neoplastic tissues, for an inhibitory
proteinaceous substance active against herpes
simplex virus (an arginine-requiring virus) re-
cently was found in extracts of Burkitt's lymphoma
cells (19). We have found that the canine LE was equally effective in interfering
with growth of canine herpesvirus (13, unpub-
lished results).

A biological role for the viral inhibitor in liver
is undetermined. However, presence of
released arginase may account for difficulties
in isolating ICH virus from triturated samples
of pathological liver material, especially if viral
titers of this organ were low and inclusions con-
stituted of 20% liver suspensions. This study also
further emphasizes the requirement for arginine
in ICH viral growth. Methionine, phenylalanine,
tryptophane, theanine, leucine, valine, and
histidine all have been reported as required for
herpes simplex virus growth in L cells (17).
Failures to find ICH or canine herpes viral inhibi-
atory activity in fractions of crude LE ob-
tained after rate zonal centrifugation in 5 to
20% sucrose gradients, other than in those with
arginase activity, suggests that the inhibition
was not a complex mixture of enzymes active
against various other amino acids.

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mining the sedimentation behavior of enzymes: application


