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Broad antiviral activity in tissues of crustaceans

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Abstract

Innate antiviral substances occur in vertebrates and may function as host defenses. Virus infections are common among invertebrates, but little is known about the ability of invertebrates to control viral infections. Pre-existing antiviral substances may be particularly important, since invertebrates lack the antiviral defense conferred by specific immunity. In our study, we found that tissue extracts of blue crab (*Callinectes sapidus*), shrimp (*Penaeus setiferus*), and crayfish (*Procambarus clarkii*) contained antiviral activities that inhibit a variety of DNA and RNA viruses, i.e. Sindbis virus (SB), vaccinia virus (VAC), vesicular stomatitis virus (VS), mengo virus (MENGO), banzi virus (BANZI) and poliomyelitis (POLIO). The concentration of inhibitory activity was relatively high, ranging from 102 to 216 U/g tissue for Sindbis virus, using the various tissue extracts. The other viruses were somewhat less sensitive to the inhibitor. The main antiviral activity in the inhibitor preparation from blue crab resided in an approximately 440 kDa fraction. It was inactivated significantly by lipid extraction, but not by proteinase K or glycosidases. The antiviral mechanism of the inhibitor from the blue crab was inhibition of virus attachment to eukaryotic cells, as evidenced by inhibitory activity at 4°C. These studies are among the first to show the existence of broadly active antiviral activities in aquatic crustaceans. These antiviral substances may function as innate host defenses in these species that lack specific antibody immunity and, therefore, merit further study. © 2000 Published by Elsevier Science B.V.

Keywords: Antiviral; Broad spectrum; Crustaceans; Innate immunity

1. Introduction

Non-specific viral inhibitors have been reported in various tissues and body fluids in vertebrates (Krizanova and Rathova, 1969; Kitamura et al., 1973; Falkler et al., 1975; Yilma et al., 1985; Anders et al., 1990; Singh et al., 1992, 1993, 1995, 1999). They are active against a variety of DNA and RNA viruses, and contain protein, lipid and carbohydrates moieties as essentially functional groups in a complex of glycoprotein or glycolipid structures (Baron et al., 1989; Singh et al., 1992,

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1995, 1999). These antiviral substances might be of significance as host defenses (Thormar et al., 1979; Anders et al., 1990; Singh et al., 1992; Baron et al., 1998). In comparison with vertebrates, invertebrates do not produce specific antibodies (Schapiro, 1975), and therefore, may rely on innate defense for host protection against viruses (Schnapp et al., 1996). Little is known about possible innate antiviral substances in invertebrates.

Viruses are the most common biological agents in the sea, they number 10 billion/l and probably infect many species (Fuhrman, 1999). Viral infections are common in crustaceans. For instance, penaeid shrimps are infected by approximately 20 viruses (Lightner, 1996; Loh et al., 1997; Nadala et al., 1998; Johnson et al., 1999), while blue crabs are infected naturally or experimentally by rotavirus, enterovirus, Newcastle disease virus and poliovirus (Kingston and Dharsana, 1977; McCumber and Clem, 1977; Hejkal and Gerba, 1981; Seidel et al., 1983). Some viruses may infect multiple crustacean species. For instance, white spot syndrome virus is transferable experimentally from crabs to shrimp (Kanchanaphum et al., 1998). Some crustacean species are reported to contain antibacterial substances able to neutralize gram-positive and gram-negative bacteria in vitro (Chisholm and Smith, 1992, 1995). A few of these antibacterial substances were found to be active against specific viruses, i.e. vesicular stomatitis virus (VSV) and influenza virus (Murakami et al., 1991). Thus, crustaceans, such as crab, shrimp and crayfish, are appropriate invertebrate subjects to study innate immunity against viruses.

2. Materials and methods

2.1. Preparation of inhibitor

Fresh specimens of blue crab (*Callinectes sapidus*), shrimp (*Penaeus setiferus*), and crayfish (*Procambarus clarkii*) were purchased in Galveston, TX. Muscle tissues from different species were dissected, mixed with Hank's balanced salt solution (1 g tissue per 3 ml HBSS) and homogenized and centrifuged at 15 000 rpm for 10 min. The supernatant was passed through a 0.22 μm sterilizing filter, aliquoted and frozen at -70°C until used. The antiviral activity of U/ml of the supernatant was then converted into that of U/g tissue. These crustacean inhibitor preparations were extensively dialyzed against HBSS using a 12–14 kDa molecular weight cut off (MWCO) membrane tubing (Spectrum, Laguna Hills, CA) before antiviral studies.

2.2. Antiviral assay

Six virus species were tested, a poxvirus, vaccinia virus (VAC) strain IHDE; an alphavirus, Sindbis virus (SB) strain EgAr 339; a flavivirus, Banzi virus (BANZI) strain SAH 336; a rhabdovirus, vesicular stomatitis virus (VS) strain Indiana and enteroviruses, poliomyelitis virus type I (POLIO) strain Mahoney and mengo virus (MENGO). Antiviral activity of the inhibitor preparations was titered by a standard plaque reduction assay (Baron and McKerlie, 1981; Singh et al., 1992), on Vero cells (African green monkey kidney cells, ATCC CRL 1587). Briefly, Vero cells (2×10^4 per well) were placed in a 96-well microtiter plate and incubated until confluent cell monolayers formed.

Samples and controls were serially diluted and challenged with 30–40 plaque forming units of viruses, and the diluted sample was retained in the overlay for antiviral assay. Antiviral activity (1 U) was defined as the reciprocal of the highest dilution of the inhibitor preparation showing 50% reduction of plaques compared to medium control. In this kind of the assay, 64 U of antiviral activity means that a sample, after a 64-fold dilution, can still reduce 30–40 plaque forming units of viruses by 50% in comparison with the medium control (Baron et al., 1998). A commercial partially purified inhibitor preparation from animal tissue (Neuramide, DIFA-Cooper, Milan, Italy) was used as a positive virus inhibitor control. As with other plaque reduction assays for antibody and interferon, titers that are >2-fold different are significant at the $P < 0.05$ level using the Student's t-test when assays are repeated 3–5 times (Grossberg et al., 1974; Singh et al., 1992).

2.3. Molecular size determination

The size of the inhibitor was determined by exclusion chromatography on Superdex G200 16/60 FPLC column (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) using sodium phosphate buffer (20 mM, pH 7.4) containing 200 mM of NaCl and 1 mM of EDTA. Samples were applied to the column and fractions were collected for antiviral assays. The column was calibrated with molecular weight markers as follows: blue dextran (2000 kDa), ferritin (440 kDa), aldolase (185 kDa), bovine serum albumin (BSA) (67 kDa) and chymotrypsinogen (25 kDa).

2.4. Proteolytic digestion, deglycosylation and lipid extraction

To determine the possible functional group (s) of the inhibitor, the effects of proteolytic digestion, deglycosylation and lipid extraction on the antiviral activity of the inhibitor preparation were studied as described previously (Singh et al., 1992, 1995; Baron et al., 1998). For proteolysis, 1 ml of the inhibitor preparation was incubated with insoluble proteinase K (1.1 U, Sigma, St. Louis, MO) attached to 4% cross-linked beaded agarose. After incubation at 37°C overnight in a rotary shaker, the insoluble enzyme was removed by centrifugation and the supernatant was assayed for antiviral activity. For deglycosylation, 1 ml of the inhibitor preparation was incubated with 100 μl of a cocktail of glycosidases composed of α-galactosidase (2.6 U/ml, Boehringer Mannheim, Indianapolis, IN), β-galactosidase (1.7 U/ml, Boehringer Mannheim), β-glucosidase (3.7 U/ml, Boehringer Mannheim), β-*N*-acetylglucosaminidase (4.9 U/ml, Sigma), endoglycosidase (1.7 U/ml, Sigma), neuraminidase (1.7 U/ml, Sigma) and amannosidase (9.8 U/ml, Sigma). The reactions were stopped by adding dithiothreitol (DTT) to a final concentration of 100 mM DTT which was subsequently removed by dialysis prior to assay for residual antiviral activity. For lipid extraction, the inhibitor preparation was extracted three times with an equal volume of *n*-butanol. Antiviral activity in the aqueous phase was assayed after evaporation of residual organic solvent in a speed vac (Savant Instruments, Hicksville, NY).

2.5. Stability

The denaturing effect of sulfhydyl and chaotropic reagents were determined by treating the inhibitor preparation with DDT (100 mM) and urea (4 M), respectively, for 4 h at room temperature. The treated samples were assayed after extensive dialysis to remove residual DTT or urea.

2.6. Mode of inhibition

Experiments to determine the possible mechanism of antiviral action were carried out as described previously (Hughes et al., 1981; Kumar et al., 1984; Singh et al., 1992, 1995; Baron et al., 1998). To test whether the inhibitor preparation was virucidal, 1 ml of the inhibitor preparation was mixed with 25 μl containing $10^{3.3}$ PFU of virus (VAC, SB or VS) and incubated for 60 min at 37°C. Subsequently, the inhibitor-virus mixture was serially diluted in two-fold steps beyond the inhibitor titer and assayed for residual virus plaques.

To determine whether the inhibitor acts during attachment to cells or later in the viral replication cycle, the inhibitory effects of the inhibitor preparation at 4 and 37°C were compared. Since virus replication at 4°C does not proceed beyond attachment because the cell membrane is quiescent, this test has traditionally been interpreted as indicating that an antiviral substance inhibits the attachment of virus to target cells. For this 4°C experiment, cold inhibitor preparation (or medium control) and viruses were added to Vero cells at 4°C for 1 h. The assay plates were maintained at 4 for 2 h and then washed three times to remove the inhibitor preparation and unabsorbed virus. The plates were overlayed with medium containing methylcellulose and incubated at 37°C until viral plaques developed. For the 37°C experiment, the procedure was identical but carried out at 37°C.

To determine whether a cellular antiviral state could be induced by the inhibitor preparation, the

inhibitor preparation was pre-incubated with Vero cells at 37°C overnight, then the cells were washed three times with media before challenging with 35 PFU of VAC, SB or VS, and incubated until plaques developed.

3. Results

3.1. Antiviral spectrum

As shown in Table 1, the crab inhibitor preparation was broadly inhibitory for the six viruses tested. These viruses represent a DNA virus, non-enveloped and enveloped RNA viruses. The titers of the inhibitor varied with the different viruses. The rank order of virus sensitivity was SB, VAC, MENGO, VS, BANZI and POLIO. We tested preliminarily the inhibitory effect of the inhibitor preparation on both cell free HIV and HIV infected CEM cells, and no antiviral activity was found. The inhibitory activity of tissue extracts from two other crustaceans, shrimp and crawfish, were found to be broadly active against the same viruses with titers comparable to that of crab inhibitor preparation (Table 1).

Confluent Vero cells were incubated with serial 2-fold dilutions of the samples or medium for 2 h, and then were decanted and incubated with new medium. The Vero cells were then stained with trypan blue at 2, 12 and 36 h, and trypan blue-stained cells were counted as dead cells. No difference was found in the percent of dead cell counted (< 10 dead cells per 2×10^4 cells) in presence and absence of the samples. In addition, Vero cells (2×10^4 per well) were mixed with samples or medium controls in serial 2-fold dilutions and incubated overnight. The percent confluentcy was determined and there was no difference between the samples and medium control. The data indicated that cell toxicity was not the cause of virus inhibition.

3.2. Molecular size

The crab inhibitor preparation was fractionated by size-exclusion chromatography on a Superdex 200 26/60 FPLC column, and individual fractions were tested for the presence of antiviral activity. As showed in Fig. 1, the major peak of inhibitory activity against Sindbis virus was found in fractions 9–11 with the highest activity in fraction 10 (44 U/ml). This corresponds to a molecular mass

Table 1
Antiviral spectrum of inhibitor preparations from crab[a] crayfish[b] and shrimp[c]

Virus group	Virus species	Antiviral activity of extracts (U/g) [d], [e]		
		Crab	Crayfish	Shrimp
DNA				
Poxvirus	Vaccinia	78 ± 45	81 ± 39	144 ± 69
RNA				
Non-enveloped enterovirus	Polio	18 ± 12	18 ± 9	12 ± 8
	Mengo	51 ± 27	39 ± 21	45 ± 21
Enveloped alphavirus	Sindbis	132 ± 42	102 ± 27	216 ± 24
Flavivirus	Banzi	24 ± 15	24 ± 12	–[f]
Rhabdovirus	Vesicular stomatitis	39 ± 24	39 ± 24	15 ± 6

[a] *C. sapidus*.
[b] *P. clarkii*.
[c] *P. setiferus*.
[d] Mean $\pm$ S.D. of 3–5 experiments. Statistically differences of >2-fold are significant at $P<0.05$ by the Student's t-test.
[e] Neuramide was used as positive control in all assays, and its titer of 64–128 (U/ml) served as indicator that the test system was sensitive.
[f] Not done.

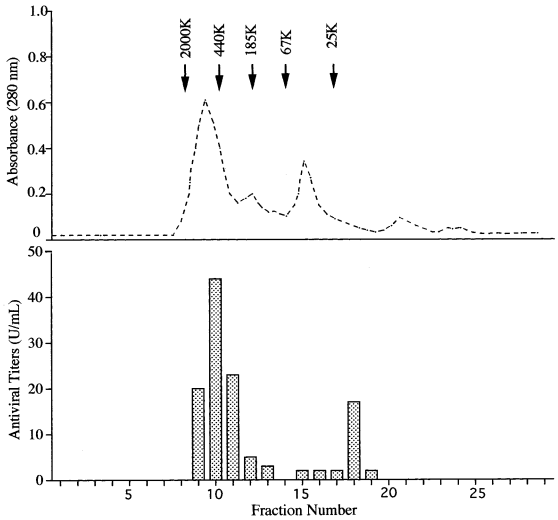

Fig. 1. Size exclusion chromatography of the inhibitor preparation of blue crab. Crab (25 ml) inhibitor preparation (39 U/ml) was concentrated five times and was loaded on a column (2.5 × 90 cm) of Superdex 200 and eluted with sodium phosphate buffer (20 mM, pH 7.4) containing 200 mM of NaCl and 1 mM of EDTA. Fractions were collected and assayed for antiviral activity. The absorbance of eluted material at 280 nm is shown on top panel, and the arrows indicate the elution time of these molecular markers used to standardize the column. Antiviral titers (U/ml) against Sindbis virus are the average of three experiments (bottom panel).

of approximately 440 kDa. The same fractions also accounted for the major inhibitory activity against VS and VAC viruses (data not shown). In addition, a secondary peak of relatively low antiviral activity (17 U/ml) was found in fractions 17–19, which corresponded to a molecular size of about 15 kDa.

3.3. Possible structure as determined by enzymatic and chemical treatments

Lipid extraction with butanol significantly decreased the antiviral activity in the residual aqueous phase of the inhibitor preparation (Table 2). Lipid extraction also resulted in the reduction of the size of the antiviral substance as evidenced by the filterability of the residual antiviral activity through a 10 K MWCO ultrafiltration membrane (data not shown). In contrast, neither insoluble proteinase K nor a cocktail of glycosidases significantly inactivated the antiviral activity of the inhibitor preparation (Table 2).

In addition, a sulfhydryl reagent (DTT) and a chaotropic reagent (urea) were used to treat the inhibitor preparation, and no loss of antiviral activity was found (Table 2). Also, the inhibitor preparation remained active against SB and VS after incubation at 37°C for 12 h.

3.4. Mode of action

To determine whether the crab inhibitor preparation was virucidal, the inhibitor preparation was

Table 2
Antiviral activity of the crab inhibitor preparation after chemical and enzymatic treatments

Treatment	Antiviral activity (U/g)[a]			
	Sindbis virus		Vesicular stomatitis virus	
	Untreated	Treated	Untreated	Treated
Proteinase K	60 ± 21	57 ± 30	78 ± 33	48 ± 33
Glycosidases	48 ± 21	60 ± 32	39 ± 18	39 ± 15
Butanol[b]	99 ± 33	15 ± 6	51 ± 27	15 ± 13
100 mM DTT[c]	69 ± 30	96 ± 48	27 ± 6	39 ± 15
4 M Urea	132 ± 39	90 ± 24	123 ± 66	99 ± 33

[a] Mean ± S.D. of 4–10 experiments, titer differences of >2-fold are significantly different ($P<0.05$).

[b] Antiviral activity in the aqueous phase of the treated sample were determined and were significantly lower than the untreated sample.

[c] Samples were incubated for 2 h with a final concentration of DTT or urea at room temperature. DTT and urea were removed by extensive dialysis after treatment.

mixed with a high dose of the viruses, incubated for 2 h and then serial two fold dilutions were made beyond the effective concentration of the inhibitor to assay for residual virus PFU. If the inhibitor preparation is virucidal, the virus plaque numbers in the higher dilutions of the mixture which are beyond the inhibitor concentration, would be much lower than that in the medium control. In our experiments, no significant difference ($P > 0.05$) of virus plaque numbers was found between the mixture with and without the inhibitor preparation, for the three viruses tested, SB ($\log_{10}$ 5.5 with and $\log_{10}$ 5.1 without sample), VAC ($\log_{10}$ 5.1; $\log_{10}$ 5.2) and VS ($\log_{10}$ 5.7; $\log_{10}$ 5.6). Thus, the inhibitor preparation appeared not to be virucidal with the viruses tested.

To determine whether the inhibitor preparation acts during virus attachment or thereafter, antiviral activity at 4 and 37°C was compared. Virus replication at 4°C does not proceed beyond attachment because the cell membrane and cell metabolism are quiescent at this temperature (Singh et al., 1995). Therefore, a comparable antiviral activity at 4 and 37°C has traditionally been interpreted as indicating that an antiviral substance inhibits the attachment of virus to target cells. Our experiments showed that the antiviral titers of the crab inhibitor preparation were at the same level ($P > 0.05$) at 4 and 37°C with the viruses tested, i.e. SB (192 ± 12 at 4°C; 327 ± 54 at 37°C), VAC (231 ± 87 at 4°C; 279 ± 72 at 37°C) and VS (144 ± 60 at 4°C; 144 ± 33 at 37°C).

To determine whether the inhibitor preparation induced durable antiviral activity in host cells, the inhibitor preparation was preincubated with Vero cells overnight and then washed before virus challenge. As shown in Table 3, the inhibitor preparation pretreated Vero cells were fully resistant to subsequent infections of SB and VAC compared with control group. This finding indicates that the crab inhibitor preparation induces a durable antiviral state in Vero cells against subsequent infection of SB and VAC. In contrast, VS was not inhibited by the same pretreated cells, indicating a reversible and probably different mechanism of inhibition.

Table 3
Cell associated antiviral activity after preincubation of cells with the crab inhibitor preparation

	Antiviral activity (U/g)[a]	
	Control	Preincubated[b]
Vaccinia	96 ± 15	48 ± 27
Sindbis	124 ± 24	96 ± 27
Vesicular stomatitis	24 ± 6	3 ± 0

[a] Mean ± S.D. of 3–4 experiments. Differences between the control and the preincubated sample are not significant statistically for VAC and SB ($P > 0.05$).

[b] Vero cells were incubated overnight with serial dilutions of the inhibitor preparation, and washed three times with medium (preincubation) or left unwashed (control) before virus challenge.

4. Discussion

In this paper, we demonstrate that tissue extracts from invertebrate crustaceans, crab, shrimp and crayfish are broadly antiviral against a variety of viruses including DNA and enveloped and non-enveloped RNA viruses. Among vertebrates, a number of non-specific inhibitors of narrow and broad antiviral spectrum have been reported in body tissues and fluids (Ginsberg, 1960; Krizanova and Rathova 1969; Kitamura et al., 1973; Yilma et al., 1985; Baron et al., 1989; Anders et al., 1990; Singh et al., 1992, 1999; Miller and Miller, 1993; Harmsen et al., 1995; Baron et al., 1998). It seems reasonable to expect non-specific inhibitors in invertebrates, since they lack specific immunity and therefore must rely on innate defenses against microbial attack (Schnapp et al., 1996). There are only a few reports describing naturally occurring antiviral substances in invertebrate animals (Riedel and Brown 1979; Masuda et al., 1992; Luo and Brown, 1993; Hiraki et al., 1997; Laille et al., 1998). However, these inhibitors are narrowly active against a few viruses. For instance, Tachyplesin, a peptide isolated from horseshoe crabs was reported to inhibit influenza A virus, vesicular stomatitis virus and human immunodeficiency virus (HIV-1), but not herpes simplex virus (HSV), adenovirus, reovirus and poliovirus (Murakami et al., 1991). An extract of silkworm faece was reported to have antiviral

activity only on enveloped viruses (Hiraki et al., 1997). Also, blue crabs were reported to clear radio-labeled viruses (bacteriophages and poliovirus) within a period of time that was too short for significant cell division or protein synthesis to occur. Therefore, the authors concluded that naturally occurring defenses exist in this species (McCumber and Clem, 1977).

Our studies focused on the large molecular size inhibitor, because the highest antiviral titers against viruses were found in the 440 kDa fraction of the crab inhibitor preparation. Therefore, the 440 kDa molecule accounts for the most of the virus inhibition of the crab inhibitor preparation. Consequently in our studies, small substances (<12 kDa) were removed by extensive dialysis using 12–14 kDa MWCO dialysis tubing before antiviral assay.

The antiviral activity of the inhibitor preparation was reduced by treatment with butanol lipid extraction, but was unaffected by treatments with DTT (a sulfhydryl reagent), urea (a protein solubilizing agent), proteinase K and carbohydrases. These data provided evidence that lipid components are important for a large portion of the antiviral activity in the inhibitor preparation. In addition, the lipid extraction appeared to reduce the size of the residual antiviral substance in the inhibitor preparation. A similar finding has been reported earlier. Lipid extraction of a large molecular size viral inhibitor from mouse and lamb brains released a low molecular weight moiety possessing broad antiviral activity (Singh et al., 1995; Baron et al., 1998). Lipids or lipid-containing compounds have been reported to be antiviral with diverse mechanisms of inhibition. For instance, lipids isolated from human milk were effective against enveloped viruses such as HIV, Herpes simplex, Vesicular stomatitis and Visna viruses, by affecting the viral envelope and causing leakage (Thormar et al., 1987). In contrast, lipoproteins in human serum were broadly active against both enveloped and non-enveloped viruses, by preventing virus attachment or penetration of target host cells (Singh et al., 1995, 1999).

The inhibitor preparation does not directly neutralize virus infectivity, as evidenced by the recovery of infectious viruses when the virus-inhibitor mixture was diluted beyond the inhibitory level. The inhibitor appears to act during the virus attachment stage, as evidenced by the equal antiviral titer of the inhibitor preparation at 4 and 37°C. If the inhibitor acts at both attachment and post-attachment stages, its titer should be significantly higher at 37 than at 4°C but it was not. Our data, therefore, suggest that the inhibitor preparation inhibits virus infection at an early stage of virus replication, probably by preventing virus attachment to Vero cells.

It is of interest to note that Vero cells pretreated with the inhibitor preparation, were resistant to subsequent infection with SB and VAC, but were sensitive to infection with VS (Table 3). This finding indicates that the crab inhibitor preparation induces a durable antiviral state to Vero cells against subsequently infection of SB and VAC. This cellular resistance could not be due to interferon, since Vero cells do not produce it (Hughes et al., 1981). It remains to be determined whether this cellular antiviral state may be due to inhibition of virus attachment, since inhibition also occurred at 4°C (see above). Among naturally occurring non-specific antiviral substances in vertebrates, only interferon induces a durable antiviral state in host cells (Kumar et al., 1984; Baron and Dianzani, 1994; Baron et al., 1989; Anders et al., 1990; Harmsen et al., 1995; Singh et al., 1999). However, a viral inhibitor from mosquitoes was reported to have cell associated antiviral activity (Luo and Brown, 1993, 1994). The mechanisms by which Vero cells gain cell-associated resistance to two of the three viruses is an interesting subject for future studies. Two actions by one inhibitor preparation has been reported for an antiviral substance, isolated from horseshoe crabs, which exerted an inhibitory effect on HIV in MT-4 cells by prevention of virus adsorption, while it also directly inactivated VS by destroying its envelope subunits (Morimoto et al., 1991; Murakami et al., 1991). However, further studies are important to define whether the extracts contain multiple inhibitors or a single inhibitor.

Overall, our findings suggest that non-specific broadly antiviral substances are present in crus-

taceans. They may be important host defense substances and thereby may contribute to survival despite continuing exposure to extremely high dose of viruses (Fuhrman, 1999). Therefore, further study of the broadly antiviral inhibitors in invertebrates seems warranted.

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