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Innate gastrointestinal immunity: characterization of broadly active viral inhibitors

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Abstract

Innate viral inhibitors that are broadly active have been characterized in the serum and the nervous system, but incompletely characterized in the gastrointestinal (GI) tract. GI preparations from porcine gastric mucosa, mouse intestine, and in neuramide (a pharmaceutical product), were examined for broad antiviral activity, molecular size and mechanism of action for comparison with the previously characterized, innate inhibitors in the serum and nervous system. The GI inhibitors were found to be active in high titers against RNA and DNA viruses, resistant to proteolysis, glycolysis, lipid extraction and possessed differing mechanisms of action. The mouse intestinal inhibitor prevented virus attachment to cells, and neuramide acted at an early post-attachment stage of virus multiplication. The porcine mucosal inhibitor acted as late as 6 h after initiation of the multiplication cycle. These broadly active GI inhibitors differed from the previously described serum inhibitor (UTIβ) high density lipoproteins (HDL) and the nervous system (NS) inhibitor by being smaller (600 ± 400 kDa) and resistant to proteinase K, glycosidases and organic solvents. The mouse intestinal inhibitor acts similarly to UTIβ and NS inhibitor by preventing attachment of virus to the cells. In comparison, the neuramide and the porcine mucosal inhibitor, like HDL, acted after attachment to the target cells. The innate nonspecific, broadly-active virus inhibitors, based on high titers and location, are considered important initial immune defense mechanisms against viral infections and thus potentially useful in medical applications.

Keywords: Intestinal virus inhibitors; Intestinal defense; Innate immunity

1. Introduction

Many nonspecific viral inhibitors from body fluids and tissues have been described, but most are active against only a few viruses (Karzon, 1956; Low and Baron, 1960; Holland, 1961; Baron et al., 1963; Krizanova and Rathova, 1969; Kitamura et al., 1973; Thiry et al., 1978; Gerna et al., 1980). Of particular interest are the broadly active viral inhibitors (Kumar et al., 1984; Baron et al., 1986, 1987, 1989; Singh et al., 1992, 1995, 1999) as they could be of importance in affording

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natural protection against many viral infections (Baron et al., 1998) and in medical use. The purpose of this study was to determine the properties of the broadly active inhibitors associated with the gastrointestinal (GI) tract, including size, structure, active chemical moiety and mechanisms of action. Other broadly active viral inhibitors have been reported in certain body tissues and fluids (Singh et al., 1992, 1995, 1999). The other inhibitors include high-density lipoprotein in plasma (Singh et al., 1999), UTIB in plasma (Singh et al., 1992) and NS inhibitor, which is distributed throughout the central and peripheral nervous systems (Singh et al., 1995). These other inhibitors are distinct from the present GI inhibitor as determined by their properties and mechanisms of antiviral action. These findings with the GI inhibitors are compared to the properties of the other innate inhibitors that were reported previously in various body tissues and fluids.

2. Materials and methods

2.1. Inhibitors

Porcine mucosal, neuramide and mouse intestinal inhibitors were used. The porcine mucosal preparation (National Formulary XII specifications) was obtained from American Laboratories (Omaha, NE). The porcine preparation was dissolved in Eagle's minimum essential medium (EMEM) at a ratio of 1.7 ml per g of original net weight. Antiviral activity is expressed as units/ml of this solution. Neuramide is a partially purified proprietary extract of gastric mucosa and milk, which is used medically to treat viral infection in humans. The starting material is clarified, heated, and extracted with lipid solvents. It was obtained from DIFA-Cooper, Milan, Italy as a clear, tan-colored solution that was not processed further before use.

The mouse intestinal inhibitor preparation was made from the intestines isolated from 20 g ICR female mice, Harlan Sprague–Dawley, Houston, TX. The intestines were washed in Hank's balanced salt solution (HBSS) and one set of samples (2 ml/g of intestine) was prepared by flushing medium through the lumen. The other set was minced, washed three times, and suspended in medium, two times the volume of their weight at 4°C for 24 h. The preparations were vortexed thoroughly and centrifuged at 2000 rev./min for 10 min. The supernatant was centrifuged at 15 000 rev./min for 20 min and filtered through 0.45- and 0.22-μm Corning 50-ml filters. The filtrate was aliquoted in 5-ml amounts and stored at −20°C until use. The two preparations had equal antiviral activity. The eluate from the minced and washed preparation was used for characterization. Thus for these initial characterization studies, centrifuged and filtered inhibitor preparations were used to initially establish their major properties. All experiments were performed at least two times and often four times.

2.2. Antiviral assay

Antiviral titers of the inhibitors were determined by the 50% virus plaque reduction assay, previously described (Baron and McKerlie, 1981). The viruses used were: a poxvirus, vaccinia (IHDE strain); a herpes virus, herpes simplex I (Heitzman strain); enteroviruses, poliomyelitis type I (Mahoney strain) and mengovirus (MV), an alphavirus, Sindbis (EgAr 339 strain) (SBV); a bunyavirus, Bunyamwera (original strain); and a rhabdovirus, vesicular stomatitis (Indiana strain) (VSV). These viruses were originally obtained from the National Institute of Health (Bethesda, MD), and have since been maintained in our laboratory (Kumar et al., 1984; Baron et al., 1986; Singh et al., 1992). Vero cells (African green monkey kidney cells, American Type Culture Collection, CCL 81) were used for assaying the inhibitors. The antiviral activity was expressed as units per ml. One unit (U) was determined as the reciprocal of the highest dilution of inhibitor preparation showing 50% inhibition of 30–40 plaque-forming units (PFU) used for challenge. All assays included at least two replicates and results were confirmed in repeated experiments. Neuramide was used as a positive virus inhibitor control in each assay.

2.3. Molecular size

Molecular sizing of neuramide and mouse intestine inhibitors was performed on soft-gel and high performance liquid chromatography (HPLC) supports. Preliminary determinations were performed on calibrated 1.0×50.0 cm Bio-Gel P-6 (Bio-Rad) columns equilibrated with 50 mM Tris–HCl, pH 7.5. UV absorbance was monitored at 280 nm, and 1.0-ml fractions were collected. Fractions were assayed for antiviral activity. These results were confirmed by HPLC on two SynChropak GPC Peptide columns (7.8×300 mm, SynChrom, Lafayette, IN) connected in series. Filtered and centrifuged samples of 0.5 ml were applied to the columns, which were run in 0.1 M sodium phosphate buffer, pH 7.2. Fractions of 1 ml were collected and assayed for antiviral activity.

2.4. Chemical composition

Chemical composition of inhibitors was investigated by lipid extraction and enzymatic degradation. To determine the effect of lipid extraction, an inhibitor sample was extracted five times with an equal amount of *n*-butanol and two times with ethyl ether (Singh et al., 1995). Organic and aqueous phases were separated, and the residual organic solvents were removed from the aqueous phase by evaporation in a Speedvac AES 1000 (Savant Instruments, Inc., Farmingdale, NY) before assaying for antiviral activity.

The effect of proteolysis was determined by using immobilized proteinase K (Sigma, St Louis, MO) (Singh et al., 1992). Briefly, 1 ml of inhibitor sample, preadjusted to pH 7.5, was incubated with 30 mg proteinase K after hydration at 37°C for 4 h in a rotary shaker. The enzyme beads were removed by centrifugation, and the supernatant was assayed for antiviral activity.

The role of complex carbohydrates as an essential structure in antiviral activity was determined by periodate oxidation and enzymatic deglycosylation (Singh et al., 1992). Briefly, the periodate oxidation was achieved by adding 0.35 M $NaIO_4$ to the inhibitor sample (pH 7.5) and incubating at 37°C for 24 h. The sample was dialyzed against a 100 molecular weight cut off (MWCO membrane) to remove $NaIO_4$ and assayed for antiviral MWCO membrane activity. Control experiments showed that IO_4, although slightly larger than 100 Dalton, did pass through the membrane entirely, presumably due to a tolerance range that included IO_4. Deglycosylation was performed by a modified technique of Bose et al. (1976) (Singh et al., 1992) by treating the inhibitor with a mixture of carbohydrases, consisting of α-galactosidase (Boehringer Mannheim, Indianapolis, IN); β-*N*-acetylglucosaminidase (Sigma, St Louis, MO); endoglycosidase (Sigma); neuraminidase (Sigma); and β-mannosidase (Sigma). The inhibitor sample preadjusted to pH 6.0 was mixed with 100 μl of the glycosidase mixture and incubated at 37°C in a rotary shaker for 24 h. To it was then added a sulfhydryl reducing agent, dithiothreitol (DTT), in a final concentration of 0.1 M, and the mixture was further incubated for 6 h. We modified the procedure by omitting DTT for the mouse intestinal inhibitor, since at this point we had found the small size of this inhibitor and unfolding of small molecule is not required for penetration by glycosidases. Samples containing DDT were dialyzed to remove the DTT and assayed for residual antiviral activity (Singh et al., 1992, 1995).

2.5. Mechanism of inhibition

The mechanism of action of inhibitors was studied by investigating whether or not the antiviral activity is due to (1) directly reversible neutralization of virions, (2) inhibition of virus attachment to cells, (3) inhibition of virus replication post-attachment to cells, or (4) induction of antiviral activity in the target cells as described below.

To test the ability of the inhibitor to irreversibly neutralize virus was determined as previously described (Kumar et al., 1984), using a plaque reduction assay with each of the viruses. Twenty-four to 64 U/ml of inhibitor and $10^{3.8}$ PFU of virus were mixed and incubated at 37°C for 2 h. The mixture was diluted beyond the inhibitory titer of the inhibitor and assayed for recovery of residual infectious virus.

The effect of the inhibitors on the attachment of Sindbis (SBV), vesicular stomatitis (VSV), and mengoviruses (MV) was examined by comparing the inhibitory titers at 4°C for 2 h (virus attachment, but not penetration, can occur at this temperature) with 37°C (virus attachment and penetration) (Kumar et al., 1984; Singh et al., 1992). The effective virus challenge was 40 PFU (MOI = 0.03) which was allowed to infect the confluent Vero cells in a 96-well microtiter plate. The cultures were then washed three times and overlaid with 50 μl of 0.5% methylcellulose in Hepes buffer and culture medium (MC). Plaques were stained with crystal violet and read on day 2.

The time during the virus growth cycle at which virus replication was inhibited was determined as described previously (Singh et al., 1995). Inhibitor (20 U/ml) was added at various times to a timed one-step growth cycle of the virus, and virus yield was determined at the end of a single cycle of growth by harvesting virus at 8 h. Synchronized initiation of virus replication (designated 0 h) was obtained by infecting cell monolayers with $10^{3.8}$ PFU (MOI = 0.16) of virus in 96-well cell culture plates at 4°C for 2 h to allow virus attachment. The cells were then washed three times with cold EMEM to remove unabsorbed virus and inhibitor and then refed with warm (37°C) inhibitor of EMEM containing 2% FBS. After incubation for a total of 8 h (a single cycle virus at 37°C replication) the cultures were stored at −70°C. The virus yield from quadruplicate wells was pooled for virus plaque assay. The virus yield was determined by titration of PFU on Vero cells.

At the virus dilutions used, inactive virus is in low concentration and the time of its production of any interferon would be too late to influence the single growth cycle.

The possible induction of an antiviral state in cells, e.g. like interferon (IFN), by inhibitors was determined as described previously (Singh et al., 1992). The confluent monolayers of Vero cells were incubated overnight with serial dilutions of the inhibitor preparation to allow any antiviral activity to develop in the cells, washed three times with EMEM to remove any non-cell associated inhibitory activity, and challenged with 40 PFU of virus. After 2 h of incubation the cells were overlaid with 0.5% MC. The 50% plaque reduction end point was calculated.

3. Results

3.1. Molecular size

All three sample types were tested on a variety of size exclusion columns. The antiviral activity consistently emerged in the column volume for most supports, indicating that the active moiety was of very small size. On calibrated P-6 columns, the activity began to separate from the salt peak, but gave inconclusive results. On the Synchropak GPC peptide HPLC columns, which are able to resolve small peptides, the neuramide, porcine mucosal, and mouse intestinal inhibitors all emerged with an apparent molecular size of $<$ 1000 Da. This is consistent with results of dialysis experiments, where the antiviral activity is unretained by all but the smallest (100 Da MWCO) membranes. Based on the GPC peptide results, we assign a tentative molecular weight of 600 ± 400 Da to the antiviral moiety in these preparations.

3.2. Antiviral spectrum

To determine the breadth of antiviral activity, the three gastrointestinal extracts were assayed for antiviral activity against five viruses. The antiviral activity of all three gastrointestinal inhibitors was broad in that all of the viruses tested, including a DNA virus and enveloped and nonenveloped RNA viruses were inhibited (Table 1). The titers of the inhibitors were substantial, ranging from 24 to 64 U/ml. Statistically, there were no significant differences in antiviral activity when the inhibitors were compared. The small molecular weight dialysates through 10 000 MW membranes had the same broad activity, but the retentates were inactive. Control preparations from kidney and liver tissue gave titers of <10 U/ml, and medium controls were negative. Thus, all three gastrointestinal extracts contained significant titers of broadly antiviral substance(s).

3.3. Chemical composition required for antiviral activity

To determine the structural composition required for antiviral activity, the extracts were treated to degrade proteins, break carbohydrate linkages, oxidize carbohydrates, or extract lipids and then the residual antiviral activity was measured. Treatment of neuramide, porcine mucosal and mouse intestine preparations, with either proteinase K, carbohydrases, sodium periodate, or extraction by lipid solvents (butanol and ethyl ether), did not affect their antiviral activity (Table 2). Since the carbohydrases would not degrade all possible sugar linkages expected to be present in the GI tract, we also treated with $NaIO_4$ as a general oxidizing agent with negative results. Previously, both treatments appropriately inactivated the other antiviral inhibitors in body tissues and fluids in our laboratory (Singh et al., 1992, 1995). These findings with the intestinal preparations suggest that protein, carbohydrate, and lipid moieties may not be required for the antiviral activity of GI inhibitors.

3.4. Mechanism of action

To determine whether the intestinal preparations directly inactivated viruses, viruses were pretreated with 24–64 U/ml of each inhibitor and then assayed for residual infectivity titer. Pretreatment of SBV, VSV, and MV with neuramide, porcine mucosal extract, and mouse intestine inhibitors did not change their infectivity titers significantly (data not shown). This indicated that they did not bind irreversibly to virions to permanently neutralize the infectivity, as would neutralizing antibody.

To determine whether the inhibitors acted during attachment, or later in the replication cycle, the inhibitory titers of inhibitors were compared at 4 and 37°C. The rationale for the temperature experiment is that at 4°C the cell membrane is physiologically inert, and hence virus replication should not proceed beyond initial attachment to the target cell. Comparable titers at 4 and 37°C, therefore, imply that an antiviral substance inhibits the attachment of virus to target cells. Significantly higher titers at 37 than at 4°C imply that the inhibitor acts at a post-attachment stage. Table 3 shows that the titers of neuramide and porcine mucosal inhibitors against SBV, VSV and MV at 4°C were significantly lower than those assayed at 37°C. This indicates that neuramide and porcine mucosal inhibitors may act by blocking viral replication at a post-attachment stage. The mouse intestine inhibitor, on the other hand, is active at 4°C, and, therefore, inhibits virus replication by preventing attachment of virions to the target cells.

To further determine when the inhibitor acted in the virus growth cycle, we carried out timed-addition of inhibitors in a single-cycle, yield-reduction experiment. Specifically, we determined virus yields after addition of inhibitor preparations to infected cell monolayers at various times after

Table 1
Antiviral spectrum of porcine mucosal, neuramide, and mouse intestinal virus inhibitors

Sample	Antiviral activity (U/ml)[a]				
	SB[b]	VS	VAC	Mengo	HSVI
Porcine mucosa[c]	64	64	64	48	96
Neuramide[c]	48	64	48	48	48
Mouse intestine[c]	24	24	32	48	32

[a] $P > 0.05$ (statistically insignificant) for the different viruses by the Student t-test. Results are the average of 4–6 experiments.
[b] Viruses: SB (Sindbis), VS (vesicular stomatitis), VAC (vaccinia), Mengo and HSVI (herpes simplex type 1).
[c] Clarified and filtered preparations without further purification, were used as described in Section 2. Neuramide was adjusted to pH 7.3 and to isotonicity by dialysis with balanced salt solution so that Neuramide would be in the same suspending medium as the other preparations.

Table 2
Glycolysis, proteolysis and lipid extraction do not inactivate the antiviral activity of porcine mucosal extract, neuramide and mouse intestine inhibitors

Sample	Antiviral activity (U/ml)[a]							
	Periodate oxidation		Glycolysis		Proteolysis		Lipid extraction	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Porcine mucosa	168	146	69	43	32	24	28	28
Neuramide	112	80	48	56	24	20	20	20
Mouse intestine	ND[b]	ND	9	17	20	19	22	15

[a] Antiviral activity was assayed against SB virus and presented as an average of at least two experiments.
[b] ND, not done; all differences not different statistically ($P>0.05$).

synchronous infection. Virus titers were measured after a single growth cycle (8 h) to avoid possible complications stemming from suppression of subsequent growth cycles by residual inhibitor. As shown in Fig. 1, when the inhibitor preparations were added at 0 h and left in the culture, they reduced the yield of SBV by 98–99%. When the inhibitors were added 1–6 h later, only the porcine mucosal inhibitor reduced the yield of SBV. Thus, the porcine mucosal inhibitor caused the inhibition of virus replication after attachment of the virus to the cell for as long as 6 h after initiation of the growth cycle. In comparison, in the presence of the mouse intestine inhibitor and the neuramide, this inhibition occurred only at 0 h, indicating inhibition either during attachment (intestinal inhibitor) or early post-attachment (neuramide). Thus, these observations are consistent with the 4°C experiment, and will be discussed later.

To determine if cells acquired resistance to virus infection after pretreatment with inhibitor, they were incubated with the inhibitor preparations (mouse intestine, neuramide and porcine mucosa) for 24 h and then washed before virus challenge. The pretreated cells did not exhibit any resistance to subsequent infection to SBV, VSV or MV (data not shown), indicating that unlike interferon, the inhibitors do not induce a durable antiviral state in the target cells.

4. Discussion

Generally in most viral infections, specific immunity is preceded by innate non-specific defenses (Baron et al., 1987, 1991). Specific immunity generally begins on day 5–7 post viral infection, but the virus multiplication starts decreasing earlier, that is, 3–5 days post infection (Baron et al., 1987, 1991). The early decrease of virus has been attributed to interferon, inflammation and innate viral inhibitors (Baron et al., 1963).

The antiviral activities of innate inhibitors against individual or families of viruses are reported to reside in a variety of uncharacterized or partly characterized substances. Most are distinguishable from antibody, interferon, and complement. Many of the antiviral substances have generally been reported to act against single viruses, such as some coronaviruses, Newcastle disease virus, variola virus, Sendai virus, and vesicular stomatitis virus (Karzon, 1956; Kitamura et al., 1973; Thiry et al., 1978; Sinibaldi et al., 1979; Gerna et al., 1980). Some are active against individual virus families, including myxoviruses, togaviruses, and retroviruses (Krizanova and Rathova, 1969; Shortridge and Ho, 1974; Welsh et al., 1976). The mechanisms of action of these viral inhibitors, when known, vary. Some of these act by irreversible neutralization of infectiv-

ity, as in the case with coronavirus and Sendai virus inhibitors (Karzon, 1956; Kitamura et al., 1973; Sinibaldi et al., 1979; Gerna et al., 1980). The myxo- and togavirus inhibitors appear to prevent hemaglutination but not infectivity (Krizanova and Rathova, 1969; Shortridge and Ho, 1974). The inhibitor of vesicular stomatitis virus acts by penetration of the viral envelope and inactivation of viral RNA (Thiry et al., 1978). The retrovirus inhibitor works via complement-mediated lysis of the virally infected cells (Welsh et al., 1976).

Among the innate non-specific viral inhibitors present in various tissues, the broadly active inhibitors are of specific interest for the present study, because they may function to prevent infection by many viruses (Singh et al., 1992; Baron et al., 1998). Some of the broadly active non-specific viral inhibitors present in plasma and nervous system from humans, rabbits and mice (Baron et al., 1986) have been characterized (Singh et al., 1992, 1995, 1999). In vivo, a protective role of the nervous tissue inhibitor against viral infection, has been shown (Baron et al., 1998). In the GI tract, an inhibitor was also found in mice, rabbits, and humans but was not fully characterized (Baron et al., 1986). The present study was done to further characterize the essential structures of the GI inhibitors from murine and porcine origin. Our studies show that mouse intestine, porcine mucosal inhibitor and neuramide preparations are active against a number of DNA and RNA viruses and have antiviral activity in the range of 24–96 U/g or ml (Table 1). They all have a molecular size of 600 ± 400 Da and appear to be resistant to lipid solvents, proteolysis and glycolysis.

The resistance to degradation by the GI inhibitors could be of significance in vivo, since to remain active in the GI tract, the inhibitors must resist the action of the various lipolytic, proteolytic and glycolytic activities of the digestive process. An interesting possibility is that antivirally defensive molecules have evolved to be resistant to digestive enzymes. Examples of proteins (Gueant et al., 1990; Festen, 1991) and carbohydrates (Engfer et al., 2000; Vonk et al., 2000) highly resistant to digestive enzymes have been reported. Other examples include IgA and vitamin B12 absorptive factors.

Our studies of antiviral mechanisms allow some limited inferences to be made, even though the GI inhibitors are impure. All these inhibitors neither directly inactivate infectious virions nor induce an antiviral state in the cells. Thus, the inhibition requires the continuous presence of inhibitor with the virus and cells. Possible inhibitory mechanisms include: (1) a low affinity binding of inhibitor to virus or cells, (2) competitive inhibition, and (3) a rapid reversibility of antiviral effects. These possibilities are under study.

The GI inhibitors appear to act at different stages of the multiplication cycle of SBV, as shown by experiments of inhibitory activities at 4°C and addition of inhibitors at different time intervals during a single-cycle of virus multiplication. The mouse intestine preparation appears to prevent virus attachment, because it is active at 4°C, a temperature at which only virus attachment may occur (Table 3). Consistent with inhibi-

Table 3
Activity of porcine mucosal, neuramide, and mouse intestinal virus inhibitors at 4°C

Sample	Antiviral activity (U/ml)[a]					
	SB[b]		VS		Mengo	
	37°C	4°C	37°C	4°C	37°C	4°C
Porcine mucosa	70	4[c]	54	3[c]	57	4[c]
Neuramide	63	<3[c]	51	<2[c]	65	<2[c]
Mouse intestine	14	8	7	8	8	14

[a] Average of more than 2–3 experiments.
[b] Viruses: SB (Sindbis), VS (vesicular stomatitis) and mengo.
[c] Statistically significantly different. $P<0.005$ using the Student's t-test comparing 4 and 37°C.

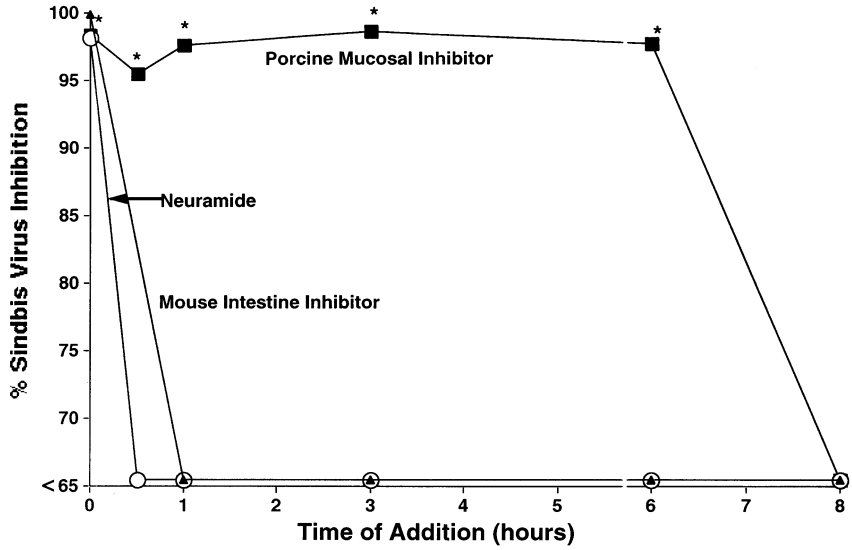

Fig. 1. Timed addition of porcine mucosal, neuramide, and mouse intestinal preparations during a single cycle replication of Sindbis virus. The inhibitor preparations were applied at various times to Vero cells after infection with 10^{4-5} PFU of Sindbis virus. The culture fluid was harvested at 8 h, the end of a single-cycle of virus multiplication. The reduction of the virus yield by different inhibitors is plotted to the level of 65%, because inhibition below this point was not statistically significant. $^{*}P < 0.05$ by the Student t-test.

tion of virus attachment by the mouse intestinal inhibitor is its very early inhibitory action only when added at 0 h during a single cycle of virus multiplication (Fig. 1).

In comparison, neuramide is not active at 4°C (Table 3), indicating that it acts at a post-attachment stage of virus multiplication. The post-attachment inhibition must be early however, because neuramide loses its inhibitory activity when added 0.5 h post-infection (Fig. 1).

The antiviral action of the porcine mucosal inhibitor appears to occur late in the virus multiplication cycle. It does not act at 4°C (Table 3), indicating that virus attachment is not inhibited. When added as late as 6 h after virus infection, it strongly inhibits virus multiplication, indicating that it acts late in the virus growth cycle. These possible mechanisms of action must eventually be confirmed using purified inhibitors and molecular probes.

Consistent with a defensive role for the GI tract inhibitors is their naturally high titer, broad antiviral activity, including an enterovirus, and location in the GI tract and its lumen. Interestingly, elution or secretion of a distinct viral inhibitor into extracellular fluids has been reported for nervous tissue (Baron et al., 1998), but has not been found using kidney and liver. To definitely assign defensive roles, future studies should show that (1) administration of these inhibitors is protective in vivo and (2) deletion of the inhibitors enhances infections.

If the GI inhibitors are host defense mechanisms then we may speculate that they may eventually be medically applicable. Such possible medical application may be made more likely by the small size of these inhibitors, which may make their synthesis more practical and also result in less antigenicity.

The present characterization of GI inhibitors allows comparison of properties of all the broadly-active antiviral innate substances reported in body fluids and tissues (Baron et al., 1986, 1987, 1989; Kumar et al., 1984; Singh et al., 1992, 1995, 1999) (Table 4). As shown in the first column of Table 4, broadly active antiviral substances are present in the plasma, nervous system, and GI tract. However, they differ in size, chemi-

Table 4
Properties of broadly antiviral innate substances

Location in situ	Inhibitor designation	Size (kDa)	Antiviral conc. range (U/ml)	Chemical structure		Mode of antiviral action	Reference
				Total	Antiviral		
Plasma	UTIβ	60	24–155	Glycoprotein	Carbohydrate	Prevents attachment	Gueant et al., 1990
Plasma	HDL	500	4–6	Lipoprotein	Protein	Post penetration	Kitamura et al., 1973; Shortridge and Ho, 1974
Nervous system	NS	4000	36–288	Lipo-glycoprotein complex	Protein and carbohydrate	Prevents attachment	Holland, 1961
Intestine	Intestinal lumen and tissues	0.6	137–2162	Undetermined[a]	Undetermined[a]	Prevents attachment	Engfer et al., 2000; see results
Gastric extract and milk	Neuramide	0.6	24–64	Undetermined[a]	Undetermined[a]	Post attachment	See results
Gastric tissues	Porcine gastric mucosa	0.6	24–64	Undetermined[a]	Undetermined[a]	Post attachment	See results

[a] Antiviral activity stable after treatment with protease, glycosidases (periodate), and lipid solvents.

cal structure, and mechanism of action. As shown in the third column, the GI inhibitors are relatively small (0.6 kDa) compared to the large molecular complexes in plasma (60 and 500 kDa) and the nervous system (4000 kDa). Interestingly, the 60 kDa molecular weight inhibitor in the plasma (UTIβ) and the 4000 kDa inhibitor in the nervous system (NS) can be broken down into active smaller size inhibitors (≤ 1 kDa) similar in size to the GI inhibitors (Baron et al., 1998; Singh et al., 1992, 1995). In addition, both small peptides and apolipoprotein A-1 from the HDL retain some antiviral activity (Srinivas et al., 1990). Thus, smaller molecular components of the larger inhibitors appear to be inhibitory.

The viral inhibitory assays indicate that all, except HDL, occur in high titers, starting at at least 25 U/ml or g (column 4). These high titers are in the same range as interferon which is an established host defense. The GI inhibitors which have the lowest molecular weight (600 Da) (columns 3), are resistant to lipid extraction, proteolytic and glycolytic enzymatic digestion (columns 5 and 6). On the other hand, the NS inhibitor is a large molecule of 4000 kDa and is inactivated by proteinase and glycosidases. HDL has a molecular size of 500 kDa and a peptide structure appears to be responsible for its antiviral activity. UTIβ is a glycoprotein of 60 kDa with carbohydrate as the antiviral moiety. The mechanism of action (column 7) of the mouse intestinal inhibitor, UTIβ, and the NS inhibitor occurs at the virus stage of attachment. The HDL inhibition is at the stage of virus penetration. Neuramide acts in the early post-attachment phase, and the porcine gastric mucosal preparation acts during the late post-attachment phase.

In conclusion, the innate broad-spectrum antiviral molecules which occur widely in the body tissues and fluids in significant titers, can be considered important as initial barriers to viral infections and thereby, also offer potential for medical use. Future studies are needed for purification and to study the molecular structures, their biosynthesis, mechanism of action, and medical application.

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References

Baron, J.L., Li, J.L., McKerlie, M.L., Shabot, J.M., Coppenhaver, D.H., 1986. A new subtype of a natural viral inhibitor (CVI) that is stable in the gastrointestinal tract. Microb. Pathog. 1 (3), 241–247.

Baron, S., McKerlie, L., 1981. Broadly active inhibitor of viruses spontaneously produced by many cell types in culture. Infect. Immun. 32 (2), 449–453.

Baron, S., Friedman, R.M., Buckler, C.E., 1963. Properties of poliovirus inhibitor from monkey brain. Proc. Soc. Exp. Med. 113, 107.

Baron, S., Dianziani, F., Stanton, G.J., Fleischmann, W.R., Jr., 1987. The interferon system: a current review to 1987. University of Texas Press.

Baron, S., Niesel, D., Singh, I.P., McKerlie, L., Poast, J., Chopra, A., Antonelli, G., Dianzani, F., Coppenhaver, D.H., 1989. Recently described innate broad spectrum virus inhibitors. Microb. Pathog. 7 (4), 237–247.

Baron, S., Tyring, S.K., Fleischmann, W.R., Jr, Coppenhaver, D.H., Niesel, D.W., Klimpel, G.R., Stanton, G.J., Hughes, T.K., 1991. The interferons. Mechanisms of action and clinical applications. JAMA 266 (10), 1375–1383.

Baron, S., Chopra, A.K., Coppenhaver, D.H., Gelman, B.B., Poast, J., Singh, I.P., 1998. A defense role of a natural antiviral substance in the nervous system. J. Neuroimmunol. 85, 168.

Bose, S., Gurari-Rotman, D., Ruegg, U.T., Corley, L., Anfinsen, C.B., 1976. Apparent dispensability of the carbohydrate moiety of human interferon for antiviral activity. J. Biol. Chem. 251 (6), 1659–1662.

Engfer, M.G., Stahl, B., Finke, B., Sawatzki, G., Daniel, H., 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am. J. Clin. Nutr. 1589–1596.

Festen, H.P., 1991. Intrinsic factor secretion and cobalamin absorption. Physiological and pathophysiology in the gastrointestinal tract. Scand. J. Gastroenterol. Suppl. 188, 1–7.

Gerna, G., Cattaneo, E., Cereda, P.M., Revelo, M.G., Achilli, G., 1980. Human coronavirus OC43 serum inhibitor and neutralizing antibody by a new plaque-reduction assay. Proc. Soc. Exp. Biol. Med. 163 (3), 360–366.

Gueant, J.L., Champigneulle, B., Gaucher, P., Nicolas, J.P., 1990. Malabsorption of vitamin B12 in the pancreatic insufficiency of the adult and of the child. Pancreas 5 (5), 559–567.

Holland, J.J., 1961. Receptor affinities as major determents of enterovirus tissue tropisms in humans. Virology 15, 312.

Karzon, D.T., 1956. Non-specific viral inactivating substance (VIS) in human and mammalian sera. J. Immunol. 76, 454.

Kitamura, T., Miyagawa, Y., Tanaka, Y., 1973. Studies on a heat-labile variola virus inhibitor in normal sera. I. Detection by the variola focus reduction assay and its general characteristics. Intervirology 1 (4), 278–287.

Krizanova, O., Rathova, V., 1969. Serum inhibitors of myxoviruses. Curr. Top. Microbiol. Immunol. 47, 125–151.

Kumar, S., McKerlie, M.L., Albrecht, T.B., Goldman, A.S., Baron, S., 1984. A broadly active viral inhibitor in human and animal organ extracts and body fluids. Proc. Soc. Exp. Biol. Med. 177 (1), 104–111.

Low, R.L., Baron, S., 1960. Poliovirus inhibitor from the central nervous system of the rhesus monkey. Science 132, 622.

Shortridge, K.F., Ho, W.K., 1974. Human serum lipoproteins as inhibitors of haemagglutination for selected togaviruses. J. Gen. Virol. 23, 113.

Singh, I.P., Coppenhaver, D.H., Chopra, A.K., Baron, S., 1992. Further characterization of a broad-spectrum antiviral substance in human serum. Viral Immunol. 5 (4), 293–303.

Singh, I.P., Chopra, A.K., Coppenhaver, D.H., Smith, E., Poast, J., Baron, S., 1995. Vertebrate brains contain a broadly active antiviral substance. Antiviral Res. 27 (4), 375–388.

Singh, I.P., Chopra, A.K., Coppenhaver, D.H., Ananatharamaiah, G.M., Baron, S., 1999. Lipoproteins account for part of the broad non-specific antiviral activity of human serum. Antiviral Res. 42 (3), 211–218.

Sinibaldi, L., De Stasio, A., Mastromarino, P., Seganti, L., Valenti, I., Orsi, N., 1979. Different sensitivity of hemagglutinating an hemolytic activities of Sendai virus to non-antibody inhibitors. Boll. Ist Sieroter. Milan. 58 (5), 365–370.

Srinivas, R.V., Birkedal, B., Owens, R.J., Anantharamaiah, G.M., Segrest, J.P., Compans, R.W., 1990. Antiviral effects of apolipoprotein A-I and its synthetic amphipathic peptide analogs. Virology 176 (1), 48–57.

Thiry, L., Cogniaux-Le Clerc, J., Content, J., Tack, L., 1978. Factors which influence inactivation of vesicular stomatitis virus by fresh human serum. Virology 87 (2), 384–393.

Vonk, R.J., Hagedoorn, R.E., de Graaff, R., Elzinga, H., Tabak, S., Yang, Y.X., Stellaard, F., 2000. Digestion of so-called resistant starch sources in the human small intestine. Am. J. Clin. Nutr. 72 (2), 432–438.

Welsh, R.M., Jr, Jensen, F.C., Cooper, N.R., Oldstone, N.B., 1976. Inactivation of lysis of oncornaviruses by human serum. Virology 74, 432.