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Mini-review

Recently described innate broad spectrum virus inhibitors

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Introduction

In recent years we have studied three broadly active viral inhibitors that are produced spontaneously in the body or in cell culture.[1–3] We tentatively have named them (a) contact-blocking viral inhibitor (CVI), (b) UTI α, and (c) UTI β (inhibitors originating from the University of Texas). These naturally produced inhibitors may serve as host defenses against a number of virus groups. Understanding the inhibitors' structures and mechanisms of antiviral action may lead to new insights into the mechanisms of virus-cell interactions and ultimately to medical use.

A number of viral inhibitors occur in the body.[4] Some inhibitors, including antibodies and interferons, are induced as a consequence of viral infection.[5] Other inhibitors occur in the uninfected host. They range from narrow to broad antiviral activity and inhibit viruses by diverse mechanisms. The three spontaneously occurring inhibitors that will be presented in this review appear structurally and functionally distinct from previously reported inhibitors[4] (and see below).

Our understanding of the differences among these three antiviral substances has grown as our investigations have progressed. Originally, some of the common attributes of three inhibitors (e.g. wide-spectrum antiviral action, blockage of attachment of many viruses to target cells, resistance to denaturation by extremes in pH) caused us to mistakenly believe that a single molecular moiety was involved in all the antiviral activities summarized here. More detailed investigations have allowed us to delineate at least three molecularly distinct inhibitors, which we describe here. Based on our current understanding, we here introduce a revised nomenclature to help clarify the discussion of these inhibitors.

The three inhibitors to be presented share one major characteristic: broad antiviral activity (Table 1). The distinguishing features of these three inhibitors are their molecular size, essential chemical composition, thermal and chemical stability, and physiologic site of occurrence. A summary of the principle characteristics of these three antiviral substances is presented in Table 2.

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Table 1 Antiviral spectrum of three naturally-occurring inhibitors

Virus	Typical antiviral titer[a] of inhibitor type		
	CVI[b]	UTI α[c]	UTI β[d]
DNA viruses			
Herpesviruses			
Herpes simplex I (HSV-I)	6	18	32
Varicella-Zoster (VZ)	—	64	—
Poxviruses			
Vaccinia	32	32	18
RNA viruses			
Picornaviruses			
Polio	16	12	24
Mengo	—	48	8
Orthomyoviruses			
Influenza A	—	128	—
Paramyxoviruses			
Newcastle disease (NDV)	<2	24	96
Reoviruses			
Rotavirus	—	4	—
Alphaviruses			
Semliki Forest	8	8	64
Sindbis	30	48	96
Flaviviruses			
Banzi	—	—	64
Bunyaviruses			
Bunyamwera	—	—	144
Rhabdoviruses			
Vesicular stomatitis (VSV)	6	24	128
Retroviruses			
HIV-1	—	20(?)	—

[a] Plaque reduction titer 50%, except hemagglutination yield reduction 50% for influenza A and reverse transcriptase assay for HIV-1. Cells in assay varied with the virus tested. — = not tested.
[b] References 1, 2, 3, 7 and unpublished observations.
[c] References 2, 9, 10, 20, 21 and unpublished observations.
[d] References 2, 9 and unpublished observations.

CVI was first detected unexpectedly during characterization of interferon preparations from tissue culture.[6,7] The interferon preparations under study contained a second inhibitor (CVI) with properties that clearly differentiated it from interferon[1,8] and other previously reported inhibitors.[3] The distinguishing properties of CVI include: inhibition of attachment of many viruses to cells; apparent molecular size of 3000–4000 kDa; essential peptide and carbohydrate structure; high stability to physical and chemical agents; reversibility of inhibition; and production in a number of cell cultures.

UTI α was discovered unexpectedly when we surveyed body fluids and tissue extracts for CVI which we had found previously in cell cultures.[2] Many body fluids and tissue extracts contained a broadly active viral inhibitor but with properties clearly distinct from those of CVI[9,10] (Table 2). Those properties which distinguished UTI α from CVI are: intracellular (in addition to broad extracellular) inhibition of influenza and varicella-zoster viruses; molecular size between 500 and 3000 Da; probable essential carbohydrate structure; extreme thermal stability; and presence in many body fluids and tissue extracts.

UTI β was discovered in human sera when it was found that the broad spectrum antiviral activity in normal human serum was distinct from CVI and from UTI α (unpublished observations). Although UTI β shares a broad antiviral spectrum with

Table 2 Characteristics of three naturally-occurring viral inhibitors

	CVI	UTI α	UTI β
Source	Tissue Culture	Tissue Fluids & Extracts	Serum
Antiviral activity	Broad, Inhibits attachment, Reversible	Broad, Attachment &/or replication, Reversible	Broad, Attachment &/or replication, Nonreversible (?)
Species specific	No	No	No
Thermal stability			
56°C	Stable	Stable	Stable
80°C	Stable	Stable	Stable[a]
100°C	Stable	Stable	Unstable
120°C	Unstable	Stable	Unstable
200°C	Unstable	Unstable	Unstable
Size	3000–4000 kDa	0.5–3 kDa	60–90 kDa
Proteolysis	Unstable	Stable	Stable
8M Urea	Stable	nd[b]	nd
DTT	Stable	nd	Stable
Lipid solvents	Stable (aqueous phase)	Stable (aqueous phase)	nd
Nucleases	Stable	Stable	nd
Periodate	Unstable	nd	Unstable
Possible composition	Carbohydrate-protein	Carbohydrate	Carbohydrate-protein

[a] Stable at 80°C for only 10 min.
[b] nd = not determined.

CVI and UTI α, it is distinguished by: molecular size of approximately 60–90 kDa; lability at 80°; conversion to a heat stable low molecular weight inhibitor by proteolysis; inactivation by mild oxidation (periodate) suggesting essential carbohydrate structure; and in preliminary experiments, antiviral activity that is only partially reversed by washing cells. Interestingly, human serum also contains a low level of a small molecular weight (approximately 3 kDa) inhibitor that may be related to the 3 kDa protease digestion product of the 60–90 kDa inhibitor.

We have begun characterizing and purifying these three inhibitors because they appear to be novel and broadly antiviral. They represent innate natural host defenses, whose mechanisms of activity may suggest new approaches to the development of antiviral compounds. Below we present the specific findings for each inhibitor.

Antiviral activity from cell culture: contact blocking viral inhibitor (CVI)

We originally identified a broadly active, spontaneously produced viral inhibitor in supernatants from a variety of cells grown in culture.[1] This inhibitor, which acted primarily by blocking the attachment of virus to target cells, was named contact-blocking viral inhibitor, or CVI. CVI is produced in various primary and secondary cell lines of human and murine origin, and continuous human, mouse, sheep, and rabbit cell lines[1] (J. Youngner, personal communication). The level of antiviral activity produced varies 40-fold between low and high producing cell lines. Primary mouse embryo fibroblasts (MEF), after several passages, have been shown to consistently produce significant CVI activity (24–64 units/ml), which has been used for partial purification and characterization of CVI.[3,8] Production of CVI is independent of cell density and subculturing protocols, except, as noted above, MEF produce CVI after several passages. Additionally, it is produced constitutively and rapidly with significant levels observed in culture supernatants from cell monolayers beginning within 1 h of washing with fresh media.[1,8] Spontaneous and continuous production is also suggested since inhibiting macromolecular synthesis by treating cells with actinomycin D or cyclohexamide blocks the appearance of the inhibitor.[1]

As shown in Table 1, CVI possesses a broad but not universal antiviral activity. Significant antiviral activity is observed against representative DNA, and enveloped and non-enveloped RNA viruses (herpes and poxviruses, picorna, alpha and rhabdoviruses). The lack of significant antiviral activity against NDV distinguishes CVI from non-specific inhibitors found in tissues and physiological fluids (UTI α and UTI β) which are discussed later in this review.

A critical difference between CVI and another broadly antiviral substance, interferon, is the absence of species specificity of CVI.[1,3] Although the relative antiviral titer of CVI from a given source differs when assayed on various cell types, CVI has been shown to be active on all heterologous cell lines tested. Interestingly, there is no obvious correlation between the relative antiviral titer observed for a cell type with heterologous CVI and its ability to produce CVI in culture.

The mechanism of action of CVI appears to be a rapidly reversible and nonprogressive extracellular inhibition of viral attachment to cell membranes. In this regard, antiviral activity is removed by washing and/or dilution and is not observed if CVI is introduced after viral infection of cells in single cycle virus growth experiments.[8] In addition, antiviral activity is not reduced by incubation with virus at 4°C.[3] These properties are consistent with a substance which displays low affinity reversible binding to viral particles or cells interfering with subsequent attachment to cell surfaces.

The chemical and physical properties of CVI are summarized in Table 2. This antiviral activity is distinct from most other broad spectrum viral inhibitors by virtue of its large molecular size. Gel filtration chromatography and ultrafiltration experiments both suggest that the antiviral component is extremely large, with an apparent molecular weight of >200 000 Da.[3] Sedimentation on discontinuous sucrose gradients yields the antiviral activity at a sedimentation rate equivalent to 3000–4000 kDa (Fig. 1). This

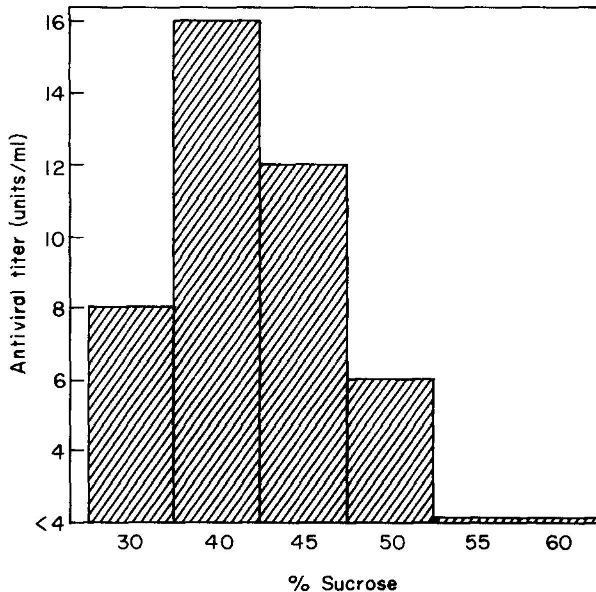

Fig. 1. Sucrose density gradient sedimentation of mouse embryo CVI. Equal volumes of mouse embryo fibroblast CVI (192 antiviral units) and 60% sucrose were mixed and applied to the top of a discontinuous sucrose gradient (30–60%) and centrifuged at 90 000 x g for 120 min. Gradient fractions (0.8 ml) were collected and dialyzed to give a final sucrose concentration of 0.6% before assay. Antiviral activity was determined against vaccinia virus on mouse L-929 cells.

surprisingly large size is not unprecedented; the Tamm-Horsfall glycoprotein was reported to be approximately 7000 kDa, although that urinary antiviral molecule was less heat stable than is CVI.[11] The sedimented CVI exhibits the same biological properties as the unsedimented material, being active against vaccinia, polio, and HSV viruses, two alphaviruses, vesicular stomatitis virus and inactive against NDV[3] (and unpublished observations). The possibility that the antiviral activity originates from shed membrane fragments has been excluded, since: (1) MEF membranes, prepared by freeze thawing and discontinuous sucrose gradients, fail to show antiviral activity; and (2) activity is not effected by extraction with lipid solvents.[3]

The physical-chemical properties of CVI are quite distinctive (Table 2). CVI possesses a thermal stability intermediate between UTI α and β, being stable at 100°C for up to 2 h but unstable at 120°C.[3] The antiviral activity is unaffected by treatment with RNase, DNase, or ether and butanol extraction, but is sensitive to proteolytic enzymes and mild oxidation with periodate. However, CVI is unaltered in molecular size or activity by a sulfhydryl reagent (dithiothreitol) and a protein solubilizing agent (8 M urea).[3] All these properties suggest that both carbohydrate and polypeptide components are required for the activity of native CVI. However, the structure of this antiviral with the surprising thermal and denaturation stability remains unknown. We have considered extracellular matrix components as the origin of CVI. However studies with related compounds like heparin and carageenan show a narrow antiviral spectrum[12] (and unpublished findings), thereby differentiating them from CVI and the other inhibitors described below.

Low molecular weight inhibitor from physiologic fluids and tissue extracts (UTI α)

A second type of broadly active virus inhibitor is found in a variety of body secretions and tissue extracts. We and others detected inhibitors with properties which match those given in Table 2 in bovine and human milk, mouse, rabbit and human gastrointestinal contents, and extracts of mouse, rabbit and human tissues[2,9,10] (unpublished results). A similar activity, uncharacterized at present, is found in human nasal and salivary secretions and liver extracts. We provisionally refer to this material as UTI α. Essential characteristics which differentiate UTI α from the tissue culture-derived inhibitor (CVI; Table 2) are that UTI α has: a smaller molecular size, extreme heat stability, lack of essential peptide structure, and a different antiviral spectrum (e.g. activity against NDV). Critical features which differentiate this material from the major inhibitor found in human serum, which is described later, are its small molecular size, extreme heat stability, and lack of essential peptide structure.

Size exclusion chromatography on soft-gel and high performance liquid chromatography (HPLC) supports has consistently yielded a molecular size in the range of 500–3000 Da[2] (unpublished results) for UTI α. The material does not contain essential peptide structure, as judged, by its resistance to digestion by trypsin, chymotrypsin, and the nonspecific proteolytic enzyme proteinase K. It is not extractable or inactivated by organic solvents, and shows extended stability to heat and pH denaturation. These and critical physical-chemical characteristics are summarized in Table 2.

Most of our preliminary work with this low molecular weight virus inhibitor from body fluids has been done with the material from bovine milk. Hence, we are particularly careful to delineate this material from other virus-inhibitory substances that have been reported from milk. The presence of antiviral lipids in milk is well documented.[13-18]

These materials can be differentiated from the low molecular weight inhibitor from tissues and body fluids in at least three areas.

(A) Only enveloped viruses are affected by antiviral milk lipids, whereas, UTI α is active against both enveloped and nonenveloped viruses;

(B) The mechanism of action of the milk lipid antivirals appears to be disruption of the lipid containing surface membrane of the enveloped viruses, leading to loss of the integrity of the virus particle and consequent loss of infectivity.[18] UTI α, in contrast, appears to function against most viruses by preventing or disrupting effective contact between the virus and target cells. It does not inactivate virus in a cell-free system.

(C) UTI α is found in the aqueous fraction of milk, not in the lipid rich cream layer. In addition, when milk whey or gastrointestinal inhibitor (UTI α) is extracted with organic solvents, the antiviral activity is not degraded, and remains in the aqueous phase.

A second virus inhibitor present in bovine milk whey[19] can also be distinguished from the antiviral substances reviewed here. This inhibitor was active when tested against rhabdoviruses, orthomyxoviruses, rhinoviruses, and reoviruses *in vitro*. It did not appear to directly inactivate virus. The activity was located in the aqueous fraction of the milk, and was not ether extractable. Significantly, however, the inhibitor was only moderately heat stable (56°C for 30 min) and was destroyed by treating at 100°C for 30 min. This is in sharp contrast to the remarkable heat stability of UTI α, which is stable for at least 2 h at 100°C and for at least 10 min at 120°C.[10] Further, we have assayed the antiviral activity of UTI α present in a proprietary pharmaceutical formulation (Neuramide; DIFA Pharmaceuticals) and in a number of bovine milk-derived proprietary infant formulas. Significant antiviral activity was detected in the aqueous fractions (e.g. 48 ± 6 anti-NDV units/ml in the infant formulas), which had undergone high heat treatment during manufacture. Additionally, UTI α is clearly not dependent on intact protein structure for its activity (Table 1), which is in contrast to the material described previously.[19] Finally, the bovine milk whey inhibitor (UTI α) passes freely through small pore (35000a minimal retention) dialysis tubing. Taken together, these characteristics serve to distinguish UTI α from other non-specific antiviral activities which have been reported from milk.

A number of biological tests have been carried out to further characterize UTI α. We have assayed inhibitor-containing preparations from human and bovine milk on cell lines of primate, rodent and avian origin. Unlike the interferons, the antiviral effect is not species specific: it is evident on all cell lines tested. However, the degree of inhibition does vary with the cell types used for assay, even when the same virus is being assayed. The activity does not produce a durable antiviral state in target cells, since the cells revert to susceptibility when the inhibitor preparation is thoroughly washed off. Preliminary studies on crude antiviral preparations from bovine milk indicate that the major antiviral effect of this inhibitor is in the prevention of attachment to the target cell of vaccinia, polio, HSV I, and ND viruses. However, different modes of action were detected in studies of VSV, varicella zoster [VZ], influenza A, and HIV. VSV was inhibited at an early, post-penetration step in the viral infection, as judged by greater inhibition of VSV at 37° vs 4°C and time of virus escape from inhibition during timed addition of inhibitor to virus infected monolayers (unpublished observations). VZ, influenza-A, and perhaps HIV, on the other hand, all appeared to be inhibited late in the replication cycle[20,21] (unpublished results). Final determination of whether these antiviral activities against viral attachement and replication represent different actions of a single inhibitor, or whether they represent different inhibitory species awaits further study using purified material.

Although the structure of UTI α has not been determined, there are a number of lines of evidence which are consistent with the active moiety containing carbohydrate or oligosaccharide structure. Negative evidence, summarized in Table 2, indicates that peptide, nucleic acid, and lipid structure is not essential to the antiviral activity of this inhibitor. Chromatographic evidence obtained during the purification of the inhibitory activity is also consistent with carbohydrate structure, i.e. the inhibitory activity has slight affinity for heparin and boronate affinity HPLC columns, and is retained by a variety of lectin affinity columns (unpublished observations). A profile of partially purified inhibitor from milk whey from an HPLC column, optimized for separation of carbohydrates, is shown in Fig. 2. The material eluting from this column represents a >95% purification of the antiviral substance, based on recovery of activity and optical density measurements. Further investigations on the structure of the active molecule are currently under way.

Broadly active viral inhibitor from human serum (UTI β)

We have also detected a third broadly active viral inhibitor, found in human serum, and have provisionally termed it UTI β. UTI β occurs in normal serum in significant titers (⩾64 U/ml) and is active against a wide range of viruses in cells of different species (Table 1). UTI β differs from CVI and UTI α in size, themostability, and chemical nature (Table 2; unpublished observations). UTI β appears to have a native molecular size in the range of 60–90 kDa, based on HPLC size exclusion chromatography, and shows moderate heat stability, being denatured by treatment at 80°C for greater than 10 min (unpublished observations). UTI β possesses a protein component which is not essential for antiviral activity, unlike UTI α which is protein free, and CVI, which has essential protein. UTI β shows antiviral activity against enveloped and non-enveloped RNA and enveloped DNA viruses (Table 1). The inhibitory activity varies with the virus used and ranges from 8 to 144 U/ml but is uniformly higher for enveloped RNA viruses.

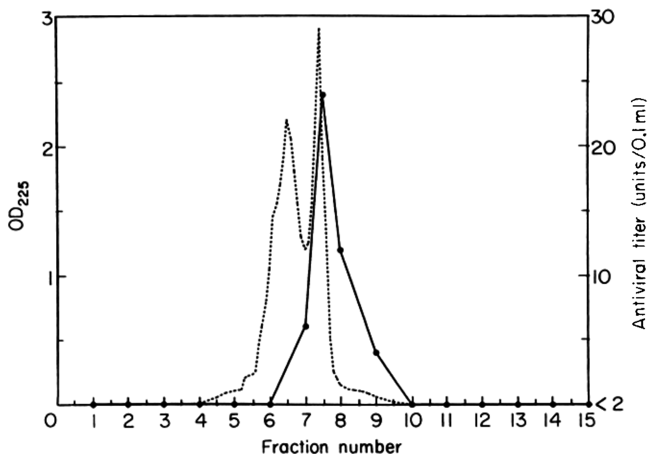

Fig. 2. Elution of bovine milk whey virus inhibitor from Ionpak KS-801 HPLC. Partially purified inhibitor from bovine milk whey was applied to a Shodex Ionpak KS-801 HPLC saccharide chromatography column (8×300 mm) outfitted with a KS-800 guard column. The column was maintained at 60°C, and developed with HPLC-grade water at a flow rate of 1 ml/min. Absorbance at 225 nm is shown by the dotted line. Fractions (0.5 min) were collected and assayed for anti-Newcastle disease virus activity by a plaque reduction assay on CER cells (solid line). Before application to this column, the milk had been defatted, deproteinized by extraction on C18 silica, desalted by chromatography on TSK 2000SW size exclusion HPLC, and passed over a heparin affinity HPLAC column.

Human serum also contains a smaller broadly active viral inhibitor, which passes through dialysis tubing of 12–14 kDa retention and constitutes about 10–20% of total virus inhibiting activity. (Approximately 80–90% of the non-specific antiviral activity is retained under the same conditions and represents the protein-bound antiviral activity of 60–90 kDa which we identify as UTI *β*.) In light of the presence of the minor, small molecular weight serum activity, we routinely remove that component before analysis of UTI *β*. Thus, in all experiments summarized here characterizing UTI *β*, serum was first exhaustively dialysed to remove the small molecular weight antiviral component.

The native UTI *β* appears to possess both protein and carbohydrate structure (in preparation). Mild oxidation with sodium metaperiodate destroys UTI *β* activity, suggesting that carbohydrate structure is essential to the antiviral activity. Proteolytic digestion consistently increases the inhibitor activity about three-fold, however (Table 3). About 90% of the antiviral activity of protease-digested UTI *β* is associated with a newly generated 1–3 kDa component(s). These data raise several possibilities about the structure of native UTI *β*. Native UTI *β* could represent a polymeric or multimeric structure. Another possibility is that the small (1–3 kDa) antiviral molecules are specifically or non-specifically bound to a carrier protein in the serum. Table 3 also shows that the protease digested activity is stable at 120°C for at least 15 min.

We have previously reported that a virus inhibitory activity from serum existed in several molecular components.[2,3] The conversion of the large size inhibitor to a smaller inhibitor with properties similar to those observed for UTI *α* is suggestive evidence that UTI *α* and *β* may be related. However, preliminary attempts to generate the small molecular weight inhibitor by disrupting the native structure of UTI *β* by sulfhydryl or chaotropic agents were unsuccessful (Table 2; unpublished observations). Nevertheless, these experiments do not eliminate the possibility that the naturally occurring small molecular weight inhibitor from sera (see above) is related to the protease-released form of UTI *β*. It may be that these molecules exist in equilibrium.

The mechanism of action of UTI *β* appears to be complex. The inhibitor does not bind tightly to virus particles, since infective virus can be recovered by simply diluting virus-inhibitor mixtures. One mode of action of native UTI *β* is the prevention of viral attachment to cells, as judged by measuring inhibition of viral infectivity during absorption of virus on to cells at 4°C.[3,9] Some viruses (e.g. HSV I) appear to be inhibited at a post-adsorption step, however. Results of preliminary experiments indicated that pretreatment of cells with native UTI *β* preparations induced a durable antiviral state against bunyamwera virus accounting for a significant portion of the antiviral activity. The generality of this result remains to be tested. It is currently unknown whether the antiviral activity represents a surface or intracellular phenomenon or whether more than one inhibitor remains in the serum preparations. The precise

Table 3 Alteration in the properties of the serum inhibitor (UTI *β*) by proteolytic treatment

	UTI *β*	
Properties	Untreated	Treated
Activity	20 U	58 U
Size	60–90 kDa	1–3 kDa
Thermostability	80°C for 10 min	120°C for 15 min

Proteinase K was added in a final concentration of 0.33 mg/ml and the mixture was incubated for four hours at 37°C. The activity was assayed by the 50% plaque reduction method on CER cells using Bunyamwera virus.

antiviral mechanisms of UTI β remain to be determined, as do the roles of the native (60–90 kDa) and protease-released (<3 kDa) forms of the antiviral molecule.

Other virus inhibitors have been reported from human serum. These include inhibitors of myxoviruses (alpha or Francis, beta or Chu, gamma, and C inhibitors of influenza, NDV, and mumps,[22,23]) poxviruses,[24] togaviruses,[25] coronavirus,[26] Sendai virus,[27] retroviruses[16] and rhabdoviruses.[28] Normal animal sera also possess a wide spectrum of viral inhibitors, many of which are homologous to those found in human sera. These inhibitors affect adeno, polio, ECHO, arbo, myxo, vaccinia, variola, Rous sarcoma, lymphocytic choriomeningitis, measles, herpes[29] vesicular stomatitis, encephalomyocarditis and caprine herpes viruses[30]; J. Youngner, personal communication).

We have evaluated the information on all of these previously described serum inhibitors. We believe that each of these inhibitors can be differentiated from UTI β based on major differences in properties such as antiviral spectrum, heat stability, chemical composition and mechanism of antiviral activity. Most of these reports describe inhibitors which either are lipid based,[25–27,29] work through direct inactivation of the virus,[22–26,28,29] are complement mediated,[16,19] are labile at 56–60°C,[16,24,29] are related to antibody,[24,29] or possess essential protein structure.[23–27,29] Although more information will be necessary for the Francis (α) influenza inhibitor to adequately compare it to UTI β, there is enough information to differentiate UTI β from most if not all previously described serum inhibitors.

Conclusions

This review presents the properties of three recently described and broadly active innate viral inhibitors. Their properties appear to distinguish them from previously described, viral inhibitors that occur in the body. One inhibitor, CVI, is produced spontaneously by a number of cell cultures, is 3000–4000 kDa and contains essential peptide and probably carbohydrate structure. The concentrations of CVI normally present in producing cultures can inhibit susceptible viruses up to 10 000-fold when low multiplicities of infection are used to infect cell cultures.[1] The second inhibitor, UTI α, is found in several body secretions and tissue extracts, is less than 3 kDa, and appears to contain essential carbohydrate structure. A third antiviral inhibitor, UTI β, is found in human serum, is approximately 60–90 kDa and is probably composed of essential carbohydrate and protein that is not essential for inhibitory activity. Proteolysis of UTI β appears to convert the inhibitor to a form that may resemble UTI α. The mechanisms of antiviral action of the three inhibitors against most viruses appear to be inhibition of the viral attachment step, but UTI α and β may also inhibit other stages in the replication of some viruses like herpesviruses and influenza virus.

The majority of contacts with viral pathogens do not lead to clinical infections. This resistance suggests possible natural defensive roles for inhibitors like UTI α and β, which is consistent with their presence at important body sites prior to and during infection, and their relatively strong antiviral effects. Further evidence for a natural defensive role would require transfer and deletion studies.

The ability of UTI α to inhibit the appearance of HIV reverse transcriptase in cell cultures deserves comment. We have looked at this question using three different experimental protocols; modest inhibition was observed in two of three experiments. Additional studies with more purified and more potent preparations are clearly indicated before final assessment can be made.

A broadly active viral inhibitor can also be found in unconcentrated urine. Although we originally thought that this inhibitor was identical to the small inhibitor from tissues and fluids[2] more detailed study indicated that inhibitors CVI, UTI α and UTI β are not

involved. Instead the inhibition correlated strongly with the concentration of ammonium ion in the urine.[31] Ammonium ions and other lysosomotropic amines are reported to inhibit the uncoating of many viruses by raising pH of the lysosomes, and thus inactivating many lysosomal enzymes.[32] The ammonium ion inhibitor was clearly distinguishable from the three inhibitors described herein based on size, antiviral spectrum and mechanism of viral inhibition.[31]

The studies on the three non-specific virus inhibitors described herein raise many questions which may be addressed in future research. To fully understand the function and mechanisms of action of these antiviral activities it will be necessary to purify and structurally analyze them. The possible relationship between the large (UTI β) and small (UTI α) inhibitors circulating in serum need to be unraveled. Do the cells that develop resistance to viral infection during incubation with UTI β require metabolic function and alteration to become protected; and is it an intracellular or cell surface event? Does the broad range of viruses affected by these substances imply that a common viral attachment pathway is being affected by these inhibitors? Does the same molecular moiety in fact affect both attachment and late replicative events of different viruses? Physiologic questions include: what are the sites of production, secretion and elimination of these inhibitors; what are the properties of the broadly active inhibitors in other body fluids and tissues (e.g. respiratory secretions, tears, saliva, liver extracts); do the levels of inhibitor change during infection? Questions of possible medical use include: can the levels of endogenous inhibitor *in vivo* be elevated sufficiently by exogenous inhibitor to protect against viral infections; and are there any side effects? Finally, concerning their defensive roles, can the inhibitors affect pathogens other than viruses?

We have presented evidence that three distinct and broadly active viral inhibitors occur spontaneously in body fluids, tissue extracts and cell cultures. They may account for some of the body's resistance to viruses and they open a number of avenues for future research.

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