Innate defences against viraemia

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SUMMARY
Human blood plasma has been reported to possess nonspecific antiviral activity. This activity is due to several preexisting naturally occurring molecules that are either active against individual members or a family of viruses. These molecules, however, have not been adequately studied to reveal their molecular structures and mechanisms of action presumably because of their low and nonspecific antiviral action. Therefore, their possible role against viraemia remains unknown. Recently, two naturally occurring nonspecific broad-spectrum antiviral agents, University of Texas Inhibitor b (UTI\textsubscript{b}) glycoprotein and high density lipoprotein, have been described in human serum. They are active against DNA and RNA viruses and one of them, UTI\textsubscript{b}, possesses significant antiviral activity of 40 units/mL. Since preexisting antiviral molecules in serum appear to be the only defence mechanisms available at the onset of viral infection they may have protective significance against viraemia. In view of this potential, we have undertaken to review the properties of these innate viral inhibitory molecules. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION
Innate nonspecific resistance mechanisms are important barriers to pathogens particularly in maintaining an initial critical balance at the onset of infections. They represent a conglomeration of mechanical barriers, cellular reactions and molecules with antimicrobial activity. Here we will consider the antiviral molecules which have been reported to occur in human serum, because viraemia is an important mode of viral spread to the target organs in the body. Normal human serum has been reported to possess inhibitory activities against particular viruses and individual groups of viruses. These antiviral activities reside in a variety of uncharacterised or partly characterised substances [1,2]. Recently certain molecules in normal human serum have been described that are active against a broad spectrum of viruses [3,4]. These molecules have been characterised for their antiviral activity, molecular structure and mechanism of antiviral action. In this review, we emphasise the properties of broadly active antiviral molecules in human serum and explore the possibility that they can be important as natural defence mechanisms.

ANTIVIRAL ACTIVITY IN HUMAN SERUM
As noted above, the innate antiviral activities reside in a variety of uncharacterised or partly characterised substances, most of which are distinguishable from antibody, interferon and complement. Many of the antiviral substances have generally been reported to act against single viruses, such as coronavirus, Newcastle disease virus, variola virus, Sendai virus and vesicular stomatitis virus [5–9]. Some are active against individual virus families, including myxoviruses, togaviruses and retroviruses [10–12]. The mechanisms of action of these viral inhibitors, when known, vary. Some of them act by irreversible neutralisation of infectivity, as in the case with coronavirus and Sendai virus inhibitors [5,8]. The myxo- and togavirus inhibitors appear to prevent haemagglutination but not infectivity [10,11]. The inhibitor of vesicular stomatitis virus acts by penetration of the viral envelope and inactivation of viral RNA [9]. The retrovirus inhibitor works

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Abbreviations used:
EMEM, Eagle’s minimum essential medium; FBS, fetal bovine serum; HBSS, Hank’s balanced salt solution; HDL, high density lipoproteins; HSV, herpes simplex virus; MDD, mean day of death; MC, methylcellulose overlay medium; MV, mengovirus; MOI, multiplicity of infection; PFU, plaque forming units; SFV, Sendikí forest virus; U, units; UTI\textsubscript{b}, University of Texas inhibitor b
via complement mediated lysis of the virally infected cells [12]. Heparin, which may be used medically to prevent clotting, has narrow antiviral activity, but deserves to be mentioned because of its potent activity against HIV-1 [13–15].

Only four broad-spectrum viral inhibitors have been reported in human sera. IFN and TNF occur in response to infections, inflammation and cancer [16–19]. The others are constitutive, i.e. UTIβ and high-density lipoprotein (HDL) and their antiviral activities are presented in Table 1 [3,4,20]. The antiviral activity was determined by a plaque reduction assay as described previously [21]. The assay was done in duplicate, using the continuous presence of 2-fold serial dilutions of the test substance in 96-well microtitre plates with confluent cell monolayer and challenged with 40 PFU of virus (MOI = 0.03). One unit (U) of antiviral activity was defined as the reciprocal of the highest dilution of the inhibitor showing 50% reduction of plaques compared with the virus control. A reference virus inhibitor standard was used in each assay as a positive control. The viruses used in these studies were originally from the National Institutes of Health, Bethesda, MD, except Coxsackie virus B3, which was obtained from Dr Charles J. Gaunt, University of Texas Health Science Center, San Antonio, TX. Vero cells (African green monkey kidney cell, American Type Culture Collection, CCL81) were used for assaying the viruses except for vaccinia. For this virus CERs (chicken embryo reticulocytes)

Table 1. Antiviral spectrum of UTIβ and HDL

| Virus                  | Antiviral titre U/mL | UTIβa | HDLb | Source       |
|------------------------|----------------------|-------|------|--------------|
| DNA viruses            |                      |       |      |              |
| Herpesviruses          |                      |       |      |              |
| Herpes simplex (HSV-1) | 36                   | 30    | 14   | Sigma Ananth  |
| Poxviruses             |                      |       |      |              |
| Vaccinia               | 24                   | 40    | 11   |              |
| RNA viruses            |                      |       |      |              |
| Picornaviruses         |                      |       |      |              |
| Polio                  | 132                  | 40    | <2   |              |
| Mango                  | 36                   | 15    | ND   |              |
| Paramyxoviruses        |                      |       |      |              |
| Newcastle disease      | 144                  | 40    | 20   |              |
| Alphaviruses           |                      |       |      |              |
| Semliki Forest         | 48                   | ND    | ND   |              |
| Sindbis                | 96                   | 24    | 14   |              |
| Flaviviruses           |                      |       |      |              |
| Banzì                  | 64                   | ND    | ND   |              |
| Bunyaviruses           |                      |       |      |              |
| Bunyamwera             | 96                   | ND    | ND   |              |
| Rhabdoviruses          |                      |       |      |              |
| Vesicular stomatitis   | 96                   | 04    | 14   |              |

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bSingh et al., 1999. Reprinted from Antiviral Research 1999; 42: 211–218, with permission from Elsevier Science.
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received from the laboratory of Dr Robert Shope, Yale University, CT at that time, were used [3,4]. A broad antiviral activity may be an important feature of an antiviral defence. Table 1 shows that UTIβ and HDL inhibited all of the viruses tested, which included DNA and enveloped and non-enveloped RNA viruses. However, UTIβ was not active against HIV (unpublished findings). The inhibitory activity of UTIβ was usually greater than HDL against the same virus, so UTIβ appears to account for most of the antiviral activity of human serum. The two preparations of HDL were comparable except that the preparation from Dr G. M. Ananthramiah, Department of Medicine, Biochemistry and Molecular Genetics and The Arthrosclerosis Research Unit, The University of Alabama at Birmingham, Birmingham, AL 35294-0012, USA, was inactive against poliovirus. This difference could be due to the extent of processing used in different methods of purification.

**MOLECULAR PROPERTIES OF UTIβ AND HDL**

**UTIβ**

As determined enzymatically UTIβ is a glycoprotein. It has a $M_r$ of approximately $60 \pm 10 \times 10^3$ based on HPLC size exclusion chromatography. Its antiviral activity is stable at pH 2–10 and at 80°C for up to 10 min. Mild oxidation by sodium periodate and glycolysis by a mixture of glycosidases destroys its antiviral activity. Proteinase digestion degrades the inhibitor into small components of $M_r < 1000$, which retain broad antiviral activity. This activity of the small components has increased heat stability (120°C for 15 min) but is still inactivated by glycosidases. Thus the antiviral activity appears to reside mainly in a separable oligosaccharide moiety of the glycoprotein, UTIβ [3].

**HDL**

Human serum HDL is a lipoprotein of $M_r$ 300 000 and occurs in the serum in the range of 30 to 80 mg/dL. Its antiviral property seems to reside in its protein component apolipoprotein A-1. Apolipoprotein A-1 has been reported to inhibit HIV and HSV-1 [22,23]. As shown in Table 1 HDL is broadly active against DNA and enveloped and non-enveloped RNA viruses.

**MECHANISMS OF ACTION**

**Inhibition of attachment**

Briefly, the mode of inhibition during virus growth was determined by investigating whether the inhibition was the result of (1) direct reversible neutralisation of virions, (2) inhibition of virus attachment to cells, (3) inhibition of viral penetration of cells or (4) effects later in the virus multiplication cycle. The reversibility of inhibition of viral infectivity was determined by mixing the inhibitor preparation with the virus suspension and then incubating the mixture at 37°C for 2 h. After incubation, the mixture was diluted beyond the inhibitory level to determine the residual titre of infective virus that was not irreversibly neutralised by the inhibitor. The results indicated that UTIβ and HDL did not irreversibly inactivate either enveloped or non-enveloped viruses.

The possibility of inhibition of virus attachment to target cells was examined by comparing the inhibitory titres at 4°C for 2 h (virus attachment only) with 37°C for 2 h (virus attachment and penetration). A virus challenge dose of 40 PFU was used to infect the confluent monolayer cells in a 96-well microtitre plate at the two temperatures. The cultures were then washed three times with Hank’s balanced salt solution (HBSS) and overlaid with 0.5% methylcellulose in Hepes buffer (MC) [24]. Plaques were stained with crystal violet and read on day 2. The rationale for this 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. Equal antiviral activity (titres) at 4°C and 37°C imply that the antiviral substance is active at 4°C, and, therefore, acts at the stage of virus attachment to target cells. Inhibitors acting at post-attachment stages result in significantly higher titres at 37°C. As shown in Table 2 the titres of UTIβ at 4°C and 37°C for all viruses used except HSV-1 were statistically indistinguishable. In the case of HSV-1, an inhibitory titre of 48 U/mL occurred at 37°C compared with 9 U/mL at 4°C, indicating that some of the inhibition of HSV-1 replication is at a step subsequent to adsorption.

**Inhibition of penetration**

In comparison, in the case of HDL, Sindbis or mengo virus attachment was not inhibited at 4°C,
indicating that HDL inhibited at a post-attachment step in virus replication. To study the possibility that the HDL prevents penetration of virus into cells, a modified method previously described was followed [23]. To allow only virus attachment, the cells were precooled to 4°C for 1 h in a 96-well microtitre plate, infected with 40 PFU (MOI = 0.03) of Sindbis or mengo virus, and after 1 h in the cold to allow virus to attach to cells, washed twice with cold HBSS to remove unabsorbed virus. The cells were warmed to 37°C to initiate virus penetration for 1 h in the presence of serial two-fold dilutions of inhibitor to allow any inhibitory effect to occur during the time of penetration. The cultures were then washed once with HBSS and refed Eagles minimum essential medium (EMEM) containing 2% fetal bovine serum (FBS). To each well 50 μL containing 100 U/mL of antibodies specific to the virus was added to neutralise any virus held extracellularly by the HDL. The cells were then incubated at 37°C for 0.5 h before washing and refeeding. Antibody controls showed the effectiveness of antibody neutralisation at 0.5 h. The cells were washed twice with HBSS, and then over layered with 0.5% MC and incubated until the plaques developed. The plaques were counted and the percent inhibition of plaques relative to control was calculated as an indicator of inhibition of virus events at the stage of virus penetration. The results in Table 3 show that cells infected in the presence of HDL consistently retained virus on their surface as evidenced by the effectiveness of neutralisation by antibody added 1 h after the start of incubation with HDL. Thus, in the presence of HDL, virus attachment occurred, but penetration did not occur.

| Virus                  | 50% Plaque reduction titre (U/mL) |
|------------------------|-----------------------------------|
| Banzi                  | 96  64                             |
| Semiliki Forest        | 32  64                             |
| Sindbis                | 64  128                            |
| Bunyamwera             | 128 144                           |
| Newcastle Disease      | 48  72                             |
| Herpes Simplex I       | 9  48                              |

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**Post-attachment inhibition by HDL**

The time of inhibition of virus replication during its growth cycle was determined as described previously [24]. The inhibitor preparation was added at various times during a one-step growth cycle of the virus and virus yield was determined during the single cycle by harvesting virus at 8 h. Synchronised initiation of virus replication (designated 0 h) was obtained by infecting monolayers with virus in 96-well culture plates at 4°C for 2 h to allow attachment of virus. The cells were then washed three times with cold EMEM to remove unabsorbed virus and inhibitor and then refed with warm (37°C) inhibitor or EMEM containing 2% FBS. After incubation for completion of a single cycle of virus replication at 8 h the cultures were stored at −70°C. For virus yield, quadruplicate wells were pooled for virus plaque assay. The virus yield was calculated from the PFU endpoints. The results are shown in Figure 1 and indicate that inhibition of Sindbis virus occurred principally when HDL was added between 0 and 1 h during the growth cycle. This early inhibition indicates that the antiviral action of HDL occurred early in the virus growth cycle and is consistent with an inhibition of viral penetration as determined in the preceding section.

**Possible induction of antiviral activity in cells**

The possible induction by the serum inhibitors of a durable antiviral state in cells (e.g. similar to IFN) by UTIβ and HDL was determined. Serial dilutions of the inhibitors and cell monolayers were incubated overnight. The cells were washed...
three times with EMEM to remove any cell associated inhibitory activity, and challenged with 40 PFU of virus. After 2 h of incubation the cultures were overlaid with 0.5% MC and incubated until the plaques formed. The 50% plaque reduction end point was calculated. The results indicated that UTI and HDL do not act by inducing a durable antiviral state in cells that would be analogous to the induction of antiviral genes and proteins by IFN.

### Table 3. HDL blocks Sindbis virus and mengovirus penetration of Vero cells

| Virus (PFU) | Temperature (°C) | Treatment 1–2 h | Antibody a | Average PFUc | Percent inhibition d |
|------------|-----------------|-----------------|------------|--------------|---------------------|
| 0–1 h      | 37              | HDLb 0–continuous | 2–3 h (37°C) | 40 + HDL | 24                   | 52e |
| 40         | 4               | HDLb 0–continuous | 2–3 h (37°C) | 40        | 34                   | 32  |
| 40         | 4               | Medium 0–continuous | 2–3 h (37°C) | 40        | 50                   | Control |
| 40         | 4               | Medium 0–continuous | 2–3 h (37°C) | 40        | 50                   | Control |
| 40 + HDL  | 37              | HDLb 0–continuous | 2–3 h (37°C) | 40        | 14                   | 60  |
| 40         | 37              | Medium 0–continuous | 2–3 h (37°C) | 40        | 35                   | Control |

**Mengovirus**

- **40** 4 HDLb 100 units/mL 24 52e
- **40** 4 HDLb Medium 34 32
- **40** 4 Medium 100 units/mL 50 Control
- **40** 4 Medium Medium 50 Control
- **40 + HDL** 37 HDLb 0–continuous — 14 60
- **40** 37 Medium 0–continuous — 35 Control

**Sindbis virus**

- **4** 4 HDLb 25 units/mL 35 27e
- **40** 4 HDLb Medium Medium 50 Control 40
- **40** 4 Medium 25 units/mL 48 Control 80
- **40** 4 Medium Medium 80 Control 48
- **40** 37 HDLb 0–continuous — 29 40
- **40** 37 Medium 0–continuous — 49 Control 49

*Antibody (Ab) was added at 1 h after treatment. Washed away after 0.5 h.*
*5 mg/mL.*
*Six to eight replicate virus plaque counts were done for each sample, then averaged.*
*Inhibition relative to medium treatment.*
*Significant compared to HDL continuous, at p < 0.0001 by the two-tail Student’s t-test.*

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Figure 1. Timed addition of HDL during a single replication cycle of Sindbis virus. A final concentration of 5 mg/mL HDL was applied at various times after virus infection with $10^3$ PFU of Sindbis virus. The culture fluid was harvested at 8 h, the end of a single cycle of virus replication. Reprinted from *Antiviral Research* 1999; 42: 211–218, with permission from Elsevier Science.
Summary of mechanisms of antiviral action

The main broad-spectrum viral inhibitors in serum are UTIβ and, to a lesser degree, HDL. Preincubation of virus with UTIβ and HDL did not reduce infectivity, indicating that they do not bind irreversibly to virions or irreversibly neutralise their infectivity. Similarly preincubation of cells with HDL and UTIβ, unlike IFN, did not induce an antiviral state in the cells. Inhibition experiments at 4°C and 37°C revealed that UTIβ inhibits virus by preventing virus attachment. This same type of experiment showed that HDL, on the other hand, acts against most viruses at a post-attachment stage. Further investigations of mechanism of viral inhibition by HDL indicated that HDL inhibits the multiplication cycle at an early stage between 0 and 1 h of initiation, most likely by preventing penetration of cell surface by virus.

IN VIVO PROTECTION AND POSSIBLE ROLE IN DEFENCE MECHANISM

In vivo studies of the protective role of these broad-spectrum serum inhibitors have not been done. There is, however, one report of in vivo protective effect of a broadly active, nonspecific virus inhibitor found in vertebrate nervous systems. Protection of inhibitor-treated mice was demonstrated against both an alphavirus and a picornavirus (Table 4). Based on this protection and other correlations, the authors suggested a natural defensive role for this broadly antiviral inhibitor that is present widely and in high concentration in tissues and extracellular fluid of human nervous system [25]. Similar in vivo protection studies should be done with the serum inhibitors.

A possible defensive role of the narrowly active serum inhibitors in natural resistance has been reviewed by Kriznova and Rathova [10]. The mechanisms of action of these inhibitors are competitive inhibition of virus attachment to cells and neutralisation of viral infectivity by a mechanism unlike classical antibody because complement is not needed for their activity. The various approaches undertaken to elucidate the possible role of these serum inhibitors in the natural resistance of the organism was grouped into three categories. In the first category an effort was made to find a correlation between the susceptibility of experimental animals to a particular virus, and the natural inhibitory activity of their sera [1,26–28]. The correlations showed that the experimental animals used were resistant to several strains of influenza virus when innate serum inhibitors against these strains were present in their sera [27].

In the second category, efforts were made to measure the effect of suppression or deletion of serum inhibitors on the susceptibility of the experimental animals to virus infection. In several experiments it was shown that mice and hamsters fed ethionine had appreciably reduced virus inhibitory activity in serum against influenza A1 and Newcastle disease viruses than did untreated.

Table 4. Inhibitor activity from human brain protects mice against Semliki Forest virus (SFV) infection or mengovirus (MV) infection

| Virus | 75% Lethal doses | Treatment | Number of mice | MDD (p value)b | % Mortality (p value)c |
|-------|------------------|-----------|----------------|----------------|-----------------------|
| SFV   | 2-8a (combined)  | Placebo   | 50             | 10.7           | 78                    |
|       |                  | HB        | 31             | 20.9 (<0.0001)d | 32 (<0.001)c          |
| MV    | 1-16a (combined) | Placebo   | 55             | 6.1            | 93                    |
|       |                  | HB        | 36             | 12.1 (<0.0002)d | 67 (<0.002)c          |

Mice were infected subcutaneously with or without the human brain (HB) inhibitor and observed for mortality and mean day of death (MDD).

aAll experiments combined.
bStudent’s t-test.
cFisher exact probability test.
dChi square test.

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controls. The ethionine treated hamsters were more susceptible to mouse adapted influenza A1 virus as indicated by titres of virus in their lungs compared with controls [29–31]. These observations point to a relationship \textit{in vivo} between the level of serum inhibitors and influenza virus multiplication and thus support the notion that the narrowly active, nonspecific viral inhibitors can be a factor in innate resistance to specific viruses.

The third category assessed the direct effect of administering the inhibitors at different times during viral infection. Experimental animals were administered an inhibitor preparation at different intervals either before or immediately after virus inoculation. Using gamma serum inhibitor it was shown that the multiplication of the inhibitor-sensitive strain of influenza A2 virus in mice lungs was inhibited. This effect was seen only when the inhibitor was administered before the virus had time to penetrate into susceptible cells. The inhibitor and the virus were administered intranasally [32–36]. Gamma inhibitor given intranasally 4–6 h post-infection or later was not protective while high doses of inhibitor given ip even at longer intervals after infection showed an appreciable protective effect [34]. While there is no satisfactory explanation for this finding, it appears that, overall, gamma inhibitor can function as a defence mechanism under some conditions.

### COMPARATIVE PROPERTIES OF BROADLY ACTIVE SERUM INHIBITORS

We have compared the properties and mechanisms of action of constitutive (UTI\(\beta\) and HDL) and inducible (IFN and TNF) inhibitors because of their importance as broadly active defences against viraemia. Table 5 compares the properties of these inhibitors and shows that constitutive virus inhibitors, UTI\(\beta\) and HDL, are clearly distinct from the inducible IFN and TNF defences.

#### CONCLUSIONS

Innate antiviral substances present in human plasma have not been carefully explored as natural defences against viraemia and infection. This review indicates that plasma contains several naturally occurring nonspecific antiviral substances, some of which are broadly active against a wide range of DNA and enveloped and non-enveloped RNA viruses. The mechanism of action of the broadly active UTI\(\beta\) occurs during virus attachment and that of HDL during virus penetration. A limited study of the \textit{in vivo} protective role of some of the antiviral substances indicates their importance against infection, particularly in early stages of infection before reactive host defences, like IFN and specific immunity, are induced. Further studies are needed in this area of virology.

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