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Lipoproteins account for part of the broad non-specific antiviral activity of human serum

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Abstract

Several antiviral substances have been detected in human serum but few have been shown to possess broad antiviral activity. These broadly active antiviral molecules could be of significance as innate defense mechanisms. We have previously identified and characterized a broadly antiviral glycoprotein, UTIβ, which accounts for 50 antiviral units/ml of human and mammalian sera. In addition there are reports of antiviral activity of human serum apolipoprotein A-1 (apo A-1), an important constituent of high density lipoprotein (HDL), against human immunodeficiency virus (HIV) and herpesvirus. Therefore we investigated (1) whether HDL is broadly antiviral, (2) how much of the broad antiviral activity of serum is due to HDL, and (3) the mechanism(s) of HDL's antiviral action. In this paper we report that (1) HDL does have broad antiviral activity, (2) HDL accounts for a modest but significant portion of the antiviral activity of serum, and (3) HDL acts by preventing virus penetration. Overall, HDL may be one of the broadly antiviral defences in the bloodstream.

Keywords: Antiviral; Apolipoprotein A-1; HDL; Lipoproteins; UTIβ

1. Introduction

Several antiviral substances have been detected in human serum but few have been shown to possess broad antiviral activity (Baron et al., 1987, 1989; Singh et al., 1992). These broadly active antiviral molecules could be of significance as innate defense mechanisms. We have previously identified and characterized a broadly antiviral glycoprotein, UTIβ, which accounts for most of the 50 antiviral units/ml of human and mammalian sera. Another serum protein, apolipoprotein (apo A-1), has been reported to inhibit human immunodeficiency virus (HIV) and herpesvirus (Owens et al., 1990; Srinivas et al., 1990). Since human apo A-1 is an important component of serum high density lipoprotein (HDL), we studied whether HDL is not only antiviral but possesses broad antiviral property, how much of

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the broad antiviral activity of serum is due to HDL, and the mechanism(s) of HDL's antiviral action. The findings show that HDL does have broad antiviral activity, HDL accounts for a modest portion of the antiviral activity of serum, and HDL acts by preventing virus penetration.

2. Materials and methods

2.1. Lipoproteins

Lipoproteins used in this study were obtained from Sigma Chemical Company, St. Louis, MO. They were lipoprotein concentrate (LP), HDL and low-density lipoprotein (LDL) from human plasma. For comparison we also used HDL prepared in the laboratory of one of us (G.M.A.).

2.2. Antiviral assay

The antiviral activity of lipoprotein preparations was determined by a plaque reduction assay as described previously (Baron and McKerlie, 1981). The assay was done in duplicate, using the continuous presence of 2-fold serial dilutions of the test substance in 96-well microtiter plates with confluent cell monolayer and challenged with 40 plaque forming units (PFU) of virus (MOI = 0.03). One unit (U) of antiviral activity was defined as the reciprocal of the highest dilution of virus inhibitor showing 50% reduction of plaques compared to virus control. A reference virus inhibitor standard was used in each assay as a positive control.

The following viruses were used: vaccinia (IHDE strain), herpes simplex (KOS strain), Newcastle disease (B-1 strain), poliomyelitis type 1 (Mahoney strain), mengo (Franklin strain), Semliki Forest (original), Sindbis (EgAr 339 strain), and vesicular stomatitis (Indiana strain). These viruses are in active use in our studies (Hughes et al., 1981; Kumar et al., 1984; Baron et al., 1998) and were originally transferred from the National Institutes of Health, Bethesda, MD. Coxsackie virus (B3) 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) (Hay et al., 1992) were used for assaying the viruses except for vaccinia. For this virus CERs (chicken embryo reticulocytes) received from Dr Robert Shope, Yale University, CT, were used (Singh et al., 1995).

2.3. Mechanism of antiviral action

The mode of viral inhibition by lipoproteins was determined by investigating whether the inhibition is the result of (1) direct reversible neutralization of virions, (2) inhibition of virus attachment to cells, or (3) inhibition of viral penetration of cells.

The reversibility of inhibition of viral infectivity was determined as previously described (Singh et al., 1995). First, 0.1 ml of the undiluted lipoprotein preparation (10 mg/ml) and 25 μl of virus suspension containing $10^{2.3-3.8}$ PFU were mixed and incubated at 37°C for 2 h. The mixture was then diluted beyond the inhibitor level to determine the residual titer of infectious virus that was not irreversibly neutralized by the lipoproteins.

The possibility that HDL prevents attachment of virus to target cells was examined by comparing the inhibitory titers at 4°C for 2 h (virus attachment only) with 37°C for 2 h (virus attachment and penetration) (Srinivas et al., 1990; Singh et al., 1995). The effective Sindbis 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 (MC) (Singh et al., 1995). Plaques were stained with crystal violet and read on day 2.

The time of inhibition of virus replication during its growth cycle was determined as described previously (Singh et al., 1995). The lipoprotein preparation was added at various times to a one step growth cycle of the virus and virus yield was determined in a single cycle growth curve by harvesting virus at 8 h. Synchronized initiation of virus replication (designated 0 h) was obtained by infecting monolayers with $10^{3.8}$ PFU (MOI = 0.16) of 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 Eagle's minimum essential medium (EMEM) to remove unabsorbed virus and inhibitor and then refed with warm (37°C) inhibitor or EMEM containing 2% fetal bovine serum (FBS). After incubation for a total of 8 h (a single cycle of virus replication) the cultures were stored at −70°C. For virus yield, quadruplicate wells were pooled for virus plaque assay. The virus yield was determined by titration of PFU on Vero cells.

To determine the possibility that HDL prevents penetration of virus into cells, a modified method of Srinivas et al. (1990) was followed. For the Vero cells precooled to 4°C for 1 h in a 96-well microtiter plate and washed twice with cold Hanks balanced salt solution (HBSS) to remove unabsorbed virus, we used an effective dose of 40 PFU of virus (MOI = 0.03). For the Vero cells held at 37°C an effective dose of 40 PFU (MOI = 0.03) was used. Serial 2-fold dilutions of HDL (starting concentration 10 mg/ml) were added and the cells were incubated at 37°C for 1 h. The cultures were then washed once with HBSS and refed EMEM containing 2% FBS. To each well 50 μl of antibodies specific to virus were added to neutralize extracellular virus, and the cells were incubated at 37°C for 0.5 h. Antibody controls showed the effectiveness of neutralization at 0.5 h. The cells were washed twice with HBSS, and then overlayered with MC and incubated until the plaques developed. The plaques were counted and the percent inhibition of plaques relative to plaques in the control was calculated.

The possible induction of a durable antiviral state in cells, e.g. like interferon, by lipoproteins was determined as described previously (Singh et al., 1995). The confluent monolayers of Vero cells were incubated overnight with serial dilutions of the lipoprotein preparation, washed three times with EMEM to remove any non-cell-associated inhibitory activity of lipoproteins, and challenged with 40 PFU of virus. After 2 h of incubation the cultures were overlaid with 0.5% MC. The 50% plaque reduction end-point was calculated. Unless stated otherwise, the data in all the tables are representative of at least three experiments for each virus.

3. Results

3.1. Breadth of antiviral activity

The broad inhibitory activity of various lipoprotein preparations against different viruses is shown in Table 1. Noteworthy is that the LP was active against both DNA and RNA viruses, and both enveloped and non-enveloped viruses. Broad antiviral activity occurred in both HDL preparations. No antiviral activity was recorded in the LDL preparation. Thus, the broad antiviral activity of LP appears to occur in its HDL component.

An interesting finding is that the difference in inhibitory activity of poliovirus by our HDL prepared by one of us (G.M.A.) and the commercial preparation may be explained by the higher purity (Anantharamaiah and Garber, 1996) of our preparation which presumably eliminated an extraneous poliovirus inhibitor (data not shown).

Table 1
Antiviral spectrum of human serum lipoproteins

Sample	MIC (mg/ml) against viruses[a]								Broad antiviral activity
	VAC	ND	VS	COX	POLIO	MENGO	SB	HS-1	
Lipoprotein[b]	0.1	0.2	–[d]	–	1.3	0.2	0.5	0.4	Yes
HDL[b]	0.3	0.3	2.7	0.3	0.3	0.8	0.5	0.4	Yes
HDL[c]	0.9	0.5	0.7	0.5	>5.5	–	0.7	0.7	Yes
LDL[b]	>1.6	>1.6	–	–	–	>1.6	>1.6	>1.6	No

[a] MIC, minimum inhibitory concentration. Viruses: VAC (vaccinia), ND (Newcastle disease), VS (vesicular stomatitis), COX (coxsackie), POLIO (polio 1), MENGO (Mengo), SB (Sindbis), HS-1 (herpes simplex 1).

[b] Sigma Chemical Co., cat. no. L5277.

[c] Prepared by Dr Anantharamaiah (Anantharamaiah and Garber, 1996).

[d] –, not done.

Table 2
Reversibility of antiviral effect of serum lipoproteins on virions

Sample (mg/ml)	Log_{10} inhibition of viruses after 2 h preincubation of viruses[a] and cells					
	MENGO	VAC	VS	ND	SB	SF
Lipoprotein[c] (8.8)	0.1	0.1	>4.4[b]	>3.8[b]	1.3[b]	1.0[b]
HDL[c] (6.0)	0.0	0.0	0.0	0.0	0.1	0.0
HDL[d] (5.0)	–[e]	–	–	–	0.0	–

[a] Viruses: MENGO (Mengo), VAC (vaccinia),VS (vesicular stomatitis), ND (Newcastle disease), SB (Sindbis), SF (Semliki Forest)
[b] $P \leq 0.05$, significant difference by Student's t-test.
[c] Sigma Chemical Co., cat. no. L5277.
[d] Prepared by Dr Anantharamaiah (Anantharamaiah and Garber, 1996).
[e] –, not done.

3.2. Mechanism of action against viruses

To determine whether various lipoproteins directly and irreversibly inactivate infectious virions, 1 mg/ml of LP or HDL was mixed with $10^{2.3-38}$ PFU and incubated at 37°C for 2 h. Thereafter, the virus–inhibitor mixture was serially diluted in 2-fold steps, beyond the 4 U/ml inhibitory titer of the LP, and HDL preparations, to determine residual virus that was not irreversibly neutralized by the inhibitor. The HDL fraction did not irreversibly inactivate either enveloped or non-enveloped viruses (Table 2). This indicates that HDL does not irreversibly bind and inactivate virus. However, the LP preparation, which contains a number of different lipoproteins, did irreversibly inactivate virions with a discrete envelope but not those viruses without a discrete envelope (mengovirus and vaccinia virus). This indicates that a non-HDL component of LP can inactivate enveloped viruses.

3.3. Mechanism of action on cells

To determine if cells acquired durable resistance to virus infection after pretreatment with lipoproteins, they were incubated with LP or HDL preparations for 24 h and then washed before virus challenge. The pretreated cells did not exhibit any resistance to subsequent infection with SBV, whereas the HDL titers in the continuous presence of LP or HDL were 12 and 24 units/ml, respectively. As expected this indicates that LP 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 interferon (Baron et al., 1987).

3.4. Mechanism of action during the replication cycle

3.4.1. Time of inhibition during growth cycle

To determine when HDL exhibits antiviral activity during the virus growth cycle, we carried out timed-addition, single cycle, yield reduction experiments. The results in Fig. 1 show that inhibition of virus multiplication occurs principally when HDL is added between 0 and 1 h during the growth cycle. This early inhibition indicates that its antiviral action occurs early in the virus growth cycle.

3.4.2. Lack of inhibition of attachment

To determine whether HDL acted during attachment or later in the replication cycle, we compared inhibitory titers during infection for 2 h at 4°C or 37°C with subsequent washing and incubation for 24 h at 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 its initial attachment to target cells. Thus, if an inhibitor is active at 4°C, then it blocks attachment. In the cases of Sindbis, vesicular stomatitis and mengo viruses, HDL did not inhibit the viruses at 4°C, indicating that HDL did not block attach-

ment in repeated experiments. Thus, HDL appears to act against most viruses at a post-attachment step.

3.4.3. Inhibition of penetration

The results of the time of addition of HDL during the virus multiplication cycle indicate that the inhibition occurs between 0 and 1 h, an early event. The lack of inhibition at 4°C indicates that early event is not attachment but it is an energy requiring process. Consequently the next step in multiplication after attachment was studied, i.e. viral penetration. The experimental design (modification of Srinivas et al., 1990) was to allow virus penetration to occur in the presence or absence of HDL for 1 h at 37°C. If the HDL prevents virus penetration then, in the presence of HDL, virus would not penetrate and would remain on the cell surface. If the HDL retains virus on the cell surface then addition of neutralizing antibodies after 1 h at 37°C should still neutralize the attached extracellular virus. In comparison, in controls not treated with HDL, the virus would penetrate cells and no longer be accessible to antibody. Virus titers under these conditions were determined as plaque forming units.

The findings shown in the representative experiments with both mengo and Sindbis viruses (Table 3) confirm that prediction. Thus, cells infected in the presence of HDL consistently retained virus on their surface as evidenced by the effectiveness of neutralization by antibody added 1 h after start of incubation with HDL. The findings were consistently reproducible and the differences were highly significant statistically. We have completed a total of six experiments, three experiments with each virus. The statistical validity for the additional experiments was as strong as the experiments presented in Table 3. Thus, HDL inhibits the penetration of the cell surface by virus.

4. Discussion

The major findings are: (1) the HDL fraction of plasma lipoprotein has broad antiviral activity, beyond the previously reported narrow activity

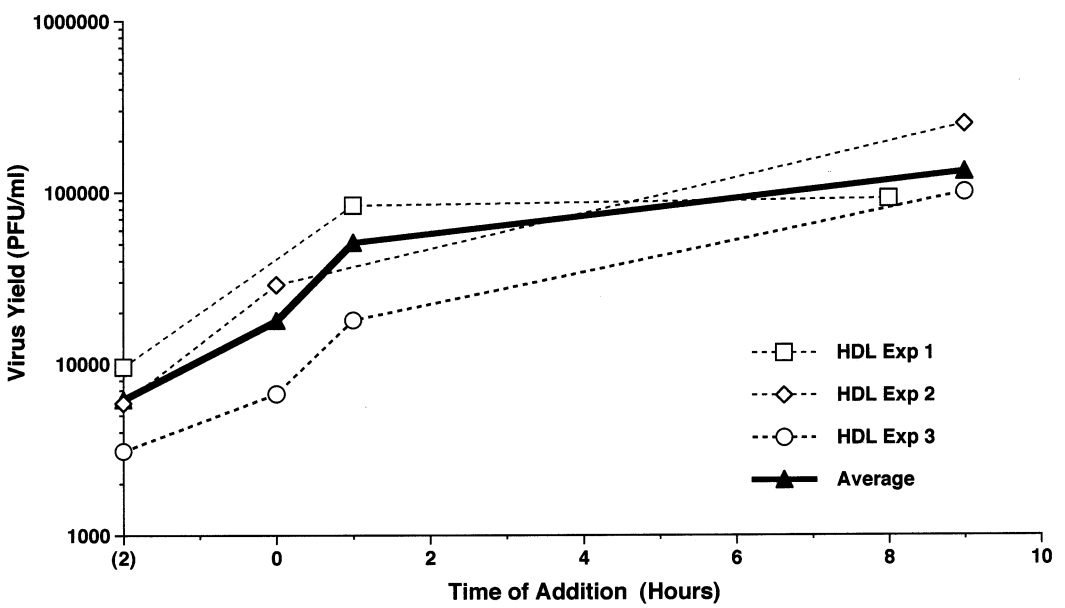

Fig. 1. Timed addition of HDL during a single replication cycle of Sindbis virus. A final concentration of 5 mg/ml HDL was applied at various times after virus infection with 10^{3-8} PFU of Sindbis virus. The culture fluid was harvested at 8 h, the end of a single cycle of virus multiplication.

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

Virus (PFU) 0–1 h	Temperature (°C)	Treatment 1–2 h (37°C)	Antibody[a] 2–3 h (37°C)	Average PFU[c] 18 h	Percent inhibition[d]
Mengo virus					
40	4	HDL[b]	100 units/ml	24	52[e]
40	4	HDL[b]	Medium	34	32
40	4	Medium	100 units/ml	50	Control
40	4	Medium	Medium	50	Control
40+HDL	37	HDL[b] 0–continuous	–	14	60
40	37	Medium 0–continuous	–	35	Control
Sindbis virus					
4	4	HDL[b]	25 units/ml	35	27[e]
40	4	HDL[b]	Medium	81	0
40	4	Medium	25 units/ml	48	Control
40	4	Medium	Medium	80	Control
40	37	HDL[b] 0–continuous	–	29	40
40	37	Medium 0–continuous	–	49	Control

[a] Antibody was added at 1 h after treatment. Washed away after 0.5 h.
[b] 5 mg/ml
[c] Six to eight replicate virus plaque counts were done for each sample, then averaged.
[d] Inhibition relative to medium treatment.
[e] Significant compared to HDL continuous, at $P<0.0001$ by the two-tail Student's t-test.

against herpes viruses and HIV; (2) the antiviral titer of HDL in blood serum averages 4 U/ml; (3) the mechanism of HDL's anti-Sindbis virus action appears to be during viral penetration; and (4) lipoproteins appear to contain additional undefined but narrowly acting inhibitor(s) which irreversibly inactivates only lipid-enveloped viruses, as previously reported for serum (Falkler et al., 1975), and also reversibly inhibits picornavirus and vaccinia virus.

A number of innate viral inhibitors have been detected in human serum (Karzon, 1956; Kriznova and Rathova, 1969; Kitamura et al., 1973; Thiry et al., 1978; Suribaldi et al., 1979; Gerna et al., 1980). Most of these inhibitors have been reported to be narrowly active against one or a few virus groups. In comparison, a broadly antiviral inhibitor (UTIβ) has been reported in human serum. It appears to be a 60 ± 10 kDa glycoprotein which has a high antiviral titer (48 U/ml) and acts by inhibiting viral attachment to cells (Singh et al., 1992). Purified HDL also exhibits broad antiviral activity but its antiviral titer in serum is one-tenth that of UTIβ. This naturally low titer of HDL may nevertheless be important as one of the host defences against viremia because this modest activity does inhibit virus yield 10–100-fold (Fig. 1); in other studies with other viruses, this degree of viral inhibition by most inhibitors is strongly correlated with in vivo protection (Baron et al., 1998). Thus, the antiviral action of HDL may contribute significantly to the broadly antiviral defense mechanism of blood.

The experiments of virus inhibition by HDL show that (1) the broad antiviral action of HDL occurs by a mechanism that does not require irreversible binding to virus or cells and (2) LP has other antiviral activities that are not broadly antiviral (Table 2). Our results indicate that a mechanism of HDL antiviral action appears to be inhibition of Sindbis viral penetration. In comparison, the other known broadly active serum inhibitor, UTIβ, appears to inhibit viral attachment (Singh et al., 1992). The evidence for HDL inhibition of penetration includes: (1) timed addition of HDL during a single cycle of virus multiplication indicates early inhibition; (2) the lack of HDL inhibition of virus at 4°C compared to 37°C

Table 4
Comparison of the non-specific antiviral activities of serum HDL, UTI-β glycoprotein, interferon and antibody

	HDL	UTI-β	Interferon	Antibody
Antiviral titer (units/ml)	4	48	Inducible	Inducible
Molecular size (kDa)	300	60	20	150
Broad antiviral activity	Yes	Yes	Yes	No
Reversible antiviral activity	Yes	Yes	No	No
Induces antiviral activity in cells	No	No	Yes	No
Mechanism of inhibition during virus multiplication cycle	Penetration	Attachment	Multiple intracellular	Extracellular neutralization

indicates that the early inhibitory mechanism is not at the attachment step; (3) neutralizing antibody inhibits virus retained on the surface of cells by the presence of HDL. These findings are in agreement with the report that apo A-1 (a major component of HDL) acts to prevent penetration by inhibiting fusion of the herpes simplex virion envelope and the cell membrane (Srinivas et al., 1990). This mechanism of action against fusion might apply to many enveloped viruses to account for their inhibition by HDL.

However, inhibition of fusion would not be expected to account for HDL inhibition of non-enveloped viruses (mengo, coxsackie and polio viruses) in our study. Consequently, we compared the inhibition of penetration of a non-enveloped virus (mengovirus) with that of the enveloped Sindbis virus. Inhibition of penetration of mengovirus occurred to the same degree as that of Sindbis virus in three replicated experiments, thereby indicating that HDL can also inhibit penetration of non-enveloped viruses. It is possible that non-enveloped picornavirus penetration of the cell membrane could be inhibited by HDL since picornaviruses attach to cells by a specific membrane receptor but then are endocytosed in a portion of the plasma membrane which is subsequently breached by uncoated virus (Yin-Murphy and Almond, 1996). This antiviral action against nonenveloped viruses is now being studied.

As an overview of broadly antiviral inhibitors in serum, Table 4 compares the properties of HDL with those of UTIβ, interferon and immunoglobulins. This comparison indicates the distinctiveness of HDL in size and mechanism of antiviral action, compared with UTIβ, interferon and immunoglobulin.

Future studies are needed to more definitively determine the in vivo host defense role of HDL and the specific molecular mechanism of HDL's inhibition of penetration.

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