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A Broadly Active Viral Inhibitor in Human and Animal Organ Extracts and Body Fluids (41918)

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Abstract. To help assess the possibility that a newly described viral inhibitor from cell cultures might play a natural defensive role *in vivo*, its distribution and concentration in human and animal organ extracts and body fluids were investigated. The concentration of the inhibitor was high in human liver, heart muscle, splenic extracts, and human serum and milk. The inhibitor in the body was indistinguishable from a previously described inhibitor produced in cell cultures that was characterized by broad antiviral activity, lack of target cell species specificity, lack of induction of stable antiviral activity in cells, rapid reversibility of antiviral action, prevention of virus attachment, and stability at 100°C. Sixteen virus plaque reduction units of the inhibitor diminished the yield of poliovirus *in vitro* by more than 1000-fold. Additional evidence that contact-blocking viral inhibitor (CVI) inhibits vaccinia virus attachment to cells is presented. A role for the inhibitor in natural defense against viral infections is possible.

Several classes of inhibitors of viral activity have been recognized *in vivo* (1–11). Recently a broadly active viral inhibitor has been detected in many cell cultures (12). The inhibitor is apparently distinct from interferon and other well-characterized viral inhibitors. This inhibitor was found to be produced spontaneously by many normal human and mouse cells in the absence of infection. It strongly inhibited the multiplication of many viruses (12), did not directly inactivate virus nor induce a stable antiviral state in cells, diminished virus attachment (13), was stable at pH 2 and 100°C and had a molecular size of about 2500 (14). The properties of this contact-blocking viral inhibitor (CVI) distinguish it from most previously described inhibitors and this comparison is summarized in the Discussion.

In view of the inhibitor's broad and potent antiviral effects *in vitro*, an *in vivo* role in defense against viral infections is possible. Such a role would require sufficient concentration of the factor at appropriate body sites. Therefore, the distribution and concentration of CVI in certain key body tissues and fluids were studied. To ascertain whether the inhibitor was CVI, and not other inhibitors (3, 5, 11–20), unique characteristics (viz broad antiviral nature, lack of target cell species specificity, lack of induction of stable antiviral activity in cells, reversible inhibition of virus multiplication by prevention of attachment, and stability at 100°C) were used to identify the antiviral factor in human and animal tissues and body fluids.

Materials and Methods. *Preparation of inhibitor samples from cell cultures, tissue extracts, and body fluids.* The cell culture inhibitor was collected from both human lung and mouse embryo culture fluids as described earlier (12). Human tissues were collected from 12-week-old fetuses. Mouse tissues were collected from 60- to 90-day-old mice. Tissue homogenizer extracts of body tissues were made as 10% suspension (w/v) using Eagles' minimum essential medium (EMEM), with 2% fetal bovine serum. The samples were clarified by centrifugation at 2000 rpm for 20 min and then stored at −20°C. Milk was collected from normal human volunteers, age 20–35 years, at 2–3 days (colostrum) or 4 weeks of lactation (mature milk). Human sera collected from 4- to 6-year-old children who had never received smallpox vaccine were kindly supplied by Dr. Q. T. Box, Department of Pediatrics, University of Texas Medical Branch. Adult sera containing antibody to vaccinia virus were also studied.

Virus. Vaccinia virus strain IHDE propagated in chick embryo cells, poliovirus type

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I Mahoney strain propagated in VERO cells, and vesicular stomatitis virus (VSV) Indiana strain propagated in chick embryo cells, were used. The stock viruses were frozen in aliquots at −70°C until used.

Titration of inhibitor samples. The titrations were done using vaccinia, polio, or VS viruses. For titration with vaccinia virus, mouse L929 cells were grown to confluency in microtest II plates (Falcon Plastics, Oxnard, Calif.). Serial dilutions of each inhibitor sample were made by mixing 0.1 ml of the sample with 0.1 ml MEM containing 2% fetal bovine serum in duplicate wells followed by addition of 25 μl of vaccinia virus containing 30–50 plaque forming units (PFU). Cultures were incubated overnight at 37°C in 4% CO_2 atmosphere. When plaques were visible microscopically, cells were stained with crystal violet. The inhibitory titer was calculated as the highest dilution of the sample which inhibited 50% of viral plaques in the continuous presence of the inhibitor (12). Titers are expressed as units/0.1 ml of original undiluted samples or as units/0.1 g of tissue specimen. Similar titrations were done using polio and VS viruses cultured in HEp-2 and WISH or mouse L cells, respectively. After 1.5-hr adsorption at 37°C, the remaining inhibitor and unadsorbed virus were decanted and the cultures overlayed with methyl cellulose. As with other plaque reduction assays for antibody and interferon (21), a significant difference in CVI titer in the same test is greater than 2-fold, and in different tests is greater than 4-fold.

To determine whether the inhibitor induced a durable antiviral effect in cells, cultures treated with inhibitor overnight were washed three times with nutrient medium before virus challenge.

Inhibition of viral adsorption at 4°C. The procedure was the same as the titration of inhibitor except that inhibitor was allowed to act on virus and cells at 4°C only during virus adsorption. Microtiter plates to which the cells were grown were cooled to 4°C for 1 hr before the test was begun. Cold inhibitor followed immediately by cold virus were added and the plates maintained at 4°C for 1.5 hr. The cells were then washed three times to remove inhibitor and unadsorbed virus before the plates were fed with medium and incubated at 37°C to permit multiplication of adsorbed virus. Control plates were treated similarly except at 37°C throughout. Inhibition following adsorption in the cold was interpreted to indicate inhibition of virus attachment since virus replication at 4°C does not proceed beyond attachment (13).

Stability at 100°C. Samples were immersed in boiling water for 30 min before assaying for inhibitor activity. Under the same conditions of temperature and high protein concentrations immunoglobulins and interferons are inactivated.

Recovery of virus from inhibitor–virus mixtures. Experiments were designed to determine whether viable virus could be recovered from inhibitor–virus mixtures by diluting the mixtures beyond the inhibitor titer. For this purpose, 0.1 ml of inhibitor sample (approximately 100 units) was added to between $10^{3.5}$ and $10^{4.5}$ PFU of poliovirus, vaccinia virus, or VSV in 0.025 ml and allowed to react for 15 min. Subsequently, 2-fold dilutions were made in microtiter cultures by serially transferring 0.1 ml of the inhibitor–virus mixture to 0.1 ml of nutrient medium in the culture wells. After making the dilutions, the cultures were overlayed as described above and incubated until virus plaques developed. Thus at dilutions greater than 100-fold, inhibitor was not active and residual virus infectivity could be detected only if virus inhibition was reversed by dilution.

Results. *CVI inhibits virus attachment to cells.* In a previous study, it was demonstrated that CVI acts on several viruses before virus penetration of cells as timed by loss of susceptability of virus to neutralization by specific antibody (13). In a more direct test the quantity of virus that attaches to cells in the presence and absence of CVI was measured. Mouse L cells were exposed to vaccinia virus at 4°C for 2 hr in the presence and absence of CVI. Input virus was varied from 736 to 8372 PFU in 4 ml of medium in a 35-mm culture dish. The low input multiplicity of infection was 0.004 to 0.0004 PFU per cell. The cells were then washed four times to remove inhibitor (12, 13) and frozen and thawed twice before being assayed for cell-associated virus titer. To achieve high accuracy, the plaque assay consisted of 2-fold serial dilutions and eight replicates per dilu-

tion. Differences of greater than 35% are significant. Controls were included to rule out virus inactivation by freezing and the presence of residual CVI. The results of the four experiments shown in Table I demonstrate highly significant ($P < 0.02$) inhibition by CVI of virus association with cells. Additionally, virus attachment in these experiments were conducted at a temperature (4°C) that does not permit virus infection to proceed past adsorption to the cell surface, thereby further supporting an inhibitory action of CVI by preventing virus attachment. The low adsorption of virus throughout these experiments is probably attributable to both the large volume of infecting medium and the 4°C temperature of adsorption.

Distribution and concentration of virus inhibitory activity in body tissues and fluids. To determine the level of virus inhibitory activity, body tissues and fluids were titrated in a plaque reduction assay against poliovirus as described under Materials and Methods. Two to six samples of each type were studied and each sample was tested for inhibitory activity several times. The results of a representative experiment are presented in Table II. High levels of antipoliovirus activity were detected in extracts of human liver, heart muscle, and spleen and also in human serum, colostrum, and mature milk. Inhibitor was not detected in extracts of human pancreas or skeletal muscle. Activity was detected in mouse liver extracts as well as in the control CVI from cultures of human fetal lung or mouse embryos. Since CVI has not yet been fully purified, it was necessary to characterize the inhibitor activity by biological and physical properties (see below).

TABLE I. EFFECT OF CVI ON VACCINIA VIRUS ATTACHMENT TO MOUSE L CELLS AT 4°C FOR 2 hr

	Cell-associated virus[b] (PFU/0.1 ml)			
	Expt 1	Expt 2	Expt 3	Expt 4
CVI concentration[a] (units/ml)				
100	6	—	—	232
20	—	6	66	—
0	24	33	112	796
Total input virus (PFU)	768	736	3328	8372

[a] CVI in mouse embryo culture fluid assayed by the vaccinia virus–mouse L-cell plaque reduction test. See Methods.

[b] Differences in cell-associated virus in the four experiments combined is significant at $P < 0.02$.

TABLE II. DETECTION OF INHIBITORY ACTIVITY IN TISSUE AS MEASURED AGAINST POLIOVIRUS EXTRACTS AND BODY FLUIDS

Sample	Units/0.1 ml of inhibitory activity[a]
Human serum	128*
Human fetal liver extract	128
Human fetal heart muscle extract	64
Human fetal spleen extract	32
Human fetal skeletal muscle extract	<8
Human fetal pancreas extract	<8
Human colostrum	128
Human mature milk	64
Mouse liver extract	16
Human fetal lung culture fluid (CVI control)	32
Mouse embryo culture fluid (CVI control)	32

[a] Inhibitory activity assayed by virus plaque reduction with poliovirus on HEp-2 cells. See Methods.

* A significant difference ($P < 0.05$) in titer is >2-fold within the same assay and >4-fold in different assays as determined by experiments similar to those done with interferon (20).

To determine the potency of the inhibitor from human liver against poliovirus, monkey VERO cells were treated with 16 virus plaque reduction units of human fetal liver inhibitor and then immediately infected with an input virus to cell ratio of 1:4. The yield of poliovirus at 24 hr was reduced to <0.01% of control. This was in agreement with previous findings of strong inhibition of VSV and sindbis virus (12).

Specific characterization of the inhibitory activity. To determine whether the inhibitor in the unpurified samples was CVI or another inhibitor, we adopted the same strategy that was successful during the early days of interferon research when only crude interferon preparations were available, i.e., characterization by a set of distinguishing properties (16). A set of molecular and biological properties unique for CVI was derived from com-

TABLE III. SPECTRUM OF ANTIVIRAL ACTIVITY AND LACK OF CELL SPECIES SPECIFICITY OF INHIBITORS IN TISSUE EXTRACTS AND BODY FLUIDS

Sample	Units/0.1 ml of inhibitory activity[a]		
Virus:	Vaccinia	VS	Polio
Cells:	Mouse L	Mouse L	Human HEp-2
Human serum	80	160	64
Human fetal liver extract	128	64	32
Human colostrum	190	200	120
Human mature milk	64	64	32
Mouse liver extract	64	32	16
Human fetal lung culture fluid (CVI control)	128	64	128
Mouse embryo culture fluid (CVI control)	128	64	64

[a] Inhibitory activity assayed by virus plaque reduction with vaccinia virus, VSV, and poliovirus on mouse L, mouse L, and human HEp-2 cells, respectively.

parisons of inhibitors described in Table I and elsewhere (1–18). We expect, as occurred with interferon, that our current studies on purification and immunization will eventually permit rapid and specific characterization of CVI. For example, in addition to the characterizations presented below, we have determined the molecular mass of the CVIs to be 2500 Da (14).

Broad antiviral activity and lack of cell species specificity. To compare the broad antiviral activity and lack of cell species specificity of a standard CVI with *in vivo* produced inhibitors, the samples were tested against vaccinia, VS and polioviruses (members of three widely different families of viruses) in cells from different species. The results of these studies are presented in Table III. The variation of inhibitory titer depending on source, virus and cell type has been reported (12). All inhibitors of body origin possessed broad antiviral activity (unlike antibody) as was seen with the CVI controls. Unlike interferon, the inhibitors also lacked cell species specificity as human samples were active when tested on mouse cells and mouse samples were active on human cells. Thus, the inhibitor in body tissue extracts and fluids, like CVI, had broad antiviral activity and lacked cell species specificity.

Lack of cell-bound virus inhibitory activity. To determine whether the inhibitor-induced antiviral activity was reversible by removal of inhibitor by washing, cultures were treated with 128 units/0.1 ml of inhibitor for 1 hr. The cells then were washed three times before adding the challenge virus. The results are presented in Table IV. Both the *in vivo* produced inhibitory and control CVI activities were completely removed by washing the assay cells indicating that there was no durable inhibitory activity associated with cells in any of the inhibitor preparations. Titer differences of ±2-fold are insignificant as indicated under Methods.

Reversible inhibition of virus multiplication

TABLE IV. REMOVAL OF INHIBITORY ACTIVITY BY WASHING INHIBITOR-TREATED CULTURES BEFORE VIRUS CHALLENGE

Sample	Units/0.1 ml of inhibitory activity[a]	
	Unwashed	Washed
Human serum	80	<20
Human fetal liver extract	128	<10
Human colostrum	80	<10
Human mature milk	160	<20
Mouse liver extract	64	<10
Human fetal lung culture fluid (CVI)	256	<10
Mouse embryo culture fluid (CVI)	256	<10

[a] Cultures were treated with 128 units/0.1 ml of inhibitor preparations for 1 hr at 37°C, washed 3×, and then infected with vaccinia virus. Inhibitory activity assayed by virus plaque reduction with vaccinia virus on mouse L cells.

TABLE V. REVERSIBILITY OF INHIBITOR ACTIVITY BY DILUTION OF VIRUS–INHIBITOR MIXTURE[a]

Sample	Virus	Titer of virus (log_{10}/0.1 ml) after mixing with medium or sample and then diluting		Difference in virus titer (log_{10})
		Medium	Sample	
Human serum (adult)	Polio	3.9	3.8	0.1
	Vaccinia	3.5	<0.0	>3.5
Human fetal liver extract	Polio	3.9	3.9	0.0
	Vaccinia	3.5	3.4	−0.1
	VS	3.6	3.8	+0.2
Human colostrum	Polio	3.9	4.0	+0.1
	VS	3.6	3.4	−0.2
Human mature milk	Polio	3.9	3.8	−0.1
	VS	3.6	3.2	−0.4
Mouse liver extract	Polio	3.9	3.9	0.0
	Vaccinia	3.5	3.4	−0.1
	VS	3.6	3.4	−0.2
Human fetal lung culture fluid (CVI control)	Polio	3.9	3.9	0.0
	Vaccinia	3.5	3.4	−0.1
	VS	3.6	3.6	0.0
Mouse embryo culture fluid (CVI control)	Polio	3.9	4.0	+0.1
	Vaccinia	3.5	3.4	−0.1
	VS	3.6	3.5	−0.1
Homologous rabbit antiserum (control)	Polio	3.9	<0.0	>3.9

[a] Basically 100 units/0.1 ml of inhibitor (or control medium) and $10^{4.0}$ PFU virus in a mixture were reacted at room temperature for 15 min, and then serially diluted beyond the inhibitor titer to assay for recoverable virus (PFU). See Methods for specifics.

by prevention of attachment. To distinguish inhibitors under study from virus inhibitors causing stable neutralization (antibody and certain lipid inhibitors) (15), the reversibility of virus inhibition by control CVI and inhibitors in extracts and body fluids was compared. The results are presented in Table V. Most *in vivo* produced inhibitors were similar to the cell culture CVI preparation in that dilution of the virus–inhibitor mixture completely reversed inhibition of virus multiplication. Only one sample (adult human serum) caused definite irreversible neutralization but only with vaccinia virus. Rabbit antiserum against poliovirus also caused the expected irreversible neutralization. This irreversible inhibition of vaccinia virus in one sample is possibly attributable to antibody because of a history of vaccination and an apparent virus-specific neutralization. Thus the adult

TABLE VI. ACTIVITY OF THE INHIBITOR AT 4 and 37°C

Sample	Units/0.1 ml of inhibitory activity[a] Preincubation temperature	
	4°C	37°C
Human serum	128	128
Human fetal liver extract	512	512
Human colostrum	128	256
Human mature milk	80	160
Mouse liver extract	64	64
Mouse embryo culture fluid (CVI)	128	128

[a] Cells kept either at 4 or 37°C for 1.5 hr with vaccinia virus and inhibitor. Cultures were then washed 3× with cold medium and incubated at 37°C until virus plaques formed.

human serum probably contained both CVI (reversible inhibition of poliovirus) and antibody (irreversible neutralization of vaccinia virus). In four sera from children without a history of vaccination, inhibition of vaccinia virus was reversible (data not presented), suggesting that CVI activity against vaccinia virus in the adult serum was masked by the presence of specific antibody.

To compare the stage at which viral replication was inhibited, viral adsorption to cultured cells in the presence of inhibitor was conducted at 4°C because this temperature does not allow virus attachment to cells to proceed beyond adsorption (see Methods). Titers for the *in vivo* produced inhibitors and CVI were statistically indistinguishable at 4 and 37°C (Table VI) indicating that virus multiplication was inhibited at the adsorption stage.

Stability at 100°C. This stability distinguishes CVI from almost all of the previously reported inhibitors of virus (14) (Table VII). The CVI-like activity in all the specimens used in the present study were repeatedly shown to be stable to 100°C for 30 min (data not presented).

Discussion. Both man and other animals are known to have a certain degree of nonspecific resistance to many viral infections (3). The mechanisms of this nonspecific resistance are, however, incompletely understood. Part of this host resistance may be due to inhibitors that are not dependent upon prior immunization or induction by viral infection. These inhibitors often can be characterized by their carbohydrate, lipid, or protein composition and by their biological properties (1–18). The recent recognition of a broadly active and potent, contact-blocking viral inhibitor (CVI) raised the possibility that this inhibitor played a role in natural resistance against viruses (12). The characteristics that distinguish CVI from previously described inhibitors are summarized in Table VII. It may be seen that CVI is distinct from most previously described inhibitors, except perhaps the incompletely characterized vaccinia virus inhibitor and the milk inhibitor.

It is recognized that to play a role in defense against viruses, an inhibitor must be present at appropriate sites and in suitable concentrations. This study was, therefore,

TABLE VII. CHARACTERISTICS THAT DISTINGUISH CVI FROM OTHER VIRAL INHIBITORS

Property	Inhibitor									
	CVI (12, 14)[a]	Antibody (15)	Interferon (16)	Myxovirus inhibitors (1, 3, 5, 6, 11)	Togavirus inhibitors (3, 5, 7, 11)	Vaccinia virus inhibitor (3, 5, 11, 17)	Paramyxovirus inhibitor (1, 3, 5, 11)	Inhibitor in milk (8)	Misc. inhibitors (2, 3, 5, 9, 11, 18)
Nonspecific inhibition of viruses	+	0	+	0	0	?	0	+	?
Low molecular weight (2500)	+	0	0	0	0	?	0	0	0 or ?
Stable at 100°C	+	0	0	0	0	?	0	0	0 or ?
Stable in lipid solvents	+	+	+	+	0	?	0	?	+ or 0
Activity in cells of heterologous species	+	+	0	+	+	+	+	+	+
Induction of stable antiviral activity in cells	0	0	+	0	0	?	0	?	?
Produced spontaneously in absence of infection	+	0	0	+	+	+	+	+	+
Reversible inhibition of viruses	+	0	0	+	+	?	?	?	?

[a] Reference numbers in parentheses.

undertaken to determine the distribution and concentration of the CVI in certain key body tissues and fluids. A virus inhibitor was detected in human serum, colostrum, mature milk, spleen, liver, and heart muscle. After detection of inhibitory activity, studies were undertaken to ascertain whether the inhibitor had the same properties as cell culture-produced CVI. The results (Tables II–VI) indicated that much of the virus-inhibitory activity in the human and mouse fetal tissues and human serum, colostrum, and mature milk was due to the same general type of inhibitor previously identified and characterized in cell cultures (12). Both inhibitors share a set of unique properties; they are broadly antiviral; lack target cell species specificity; do not induce a sustained antiviral effect in cells; act by preventing adsorption of virus; inhibit virus in a reversible fashion; and are stable at 100°C. Insufficient information concerning the previously described inhibitors from cell cultures (3, 5, 11, 17) and milk (8) is available to make definitive comparisons with CVI.

The origin of CVI in tissue extracts and body fluids has not been established. Many cultured cells produce and release CVI (12) and production by cells *in vivo* seems to be a likely source. CVI in serum could account for at least some of the CVI detected in tissue extracts. However, the absence of CVI in skeletal muscle and pancreas, which may be expected to have the same serum content as the other tissues, suggests that serum CVI may not account for all the CVI in tissue extracts. The origin and distribution of CVI in the body requires further study.

The hypothesis that CVI is part of the body's natural defense against viral infections is supported by its occurrence at key sites in the body. CVI in colostrum and milk could provide protection for the alimentary tract of the nursing infant. Furthermore, to cause disease many viruses must pass through the blood stream and the spleen which also contain CVI. It seems logical then that mucosal secretions might also possess this activity. Our recent studies have detected CVI in many body surface fluids (in preparation). Related inhibitors have been found by others in animal sera and cell cultures (Ref. (19); Yilma, 1983, submitted; Youngner, personal communication).

The potency of CVI was shown in previous studies (12) using mouse CVI to protect against VS and Sindbis viruses. It was shown that even eight virus plaque reduction units of CVI were sufficient to reduce the virus yield by at least 1000-fold in *in vitro* multicycle growth experiments. In the present study, 16 units of CVI in human fetal liver extract was found to reduce poliovirus yield by at least 1000-fold in multicycle *in vitro* growth experiments. Thus the quantity of CVI in body sites seems to be consistent with sufficient inhibition of virus multiplication to associate CVI with natural defenses.

Taken together, the present and previous studies of CVI are consistent with a possible role of CVI in natural defense against viruses because of its sufficient potency, broad spectrum of activity, and presence in protective concentrations at key body sites.

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