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Size and Stability of a Naturally Occurring Virus Inhibitor

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We recently described a virus inhibitor (contact-blocking virus inhibitor) which was produced spontaneously by untransformed human and murine cells in tissue culture (S. Baron and L. McKerlie, Infect. Immun. 32:449–453, 1981). This contact-blocking virus inhibitor was characterized by broad antiviral activity, high potency, and reversible inhibition of viral attachment. Unlike interferon, the antiviral activity of the contact-blocking virus inhibitor is not species specific. An inhibitor with similar properties can also be demonstrated in many body fluids and surface secretions. We report here studies on the stability of the antiviral species which indicate that it is resistant to denaturation by heat (100°C), acid (pH 2), and alkali (pH 12). The antiviral activity against all viruses tested resides in a low-molecular-weight molecule. The range of characteristics so far determined for the contact-blocking virus inhibitor distinguishes it from other virus inhibitors reported in the literature.

We recently described a virus inhibitor produced by many cell types in culture (1). This inhibitor has an unusual array of characteristics which taken together distinguish it from other naturally occurring virus inhibitors. Characteristics which serve to distinguish this contact-blocking virus inhibitor (CVI) include (i) lack of species specificity, (ii) broad antiviral activity, (iii) absence of tight binding by the inhibitor to the virus, (iv) probable peptide nature of the active component, and (v) action of the inhibitor through prevention of virus attachment (1, 4). Subsequently, it has been determined that CVI is a naturally occurring antiviral substance and is normally present in a number of physiological fluids, including human and bovine milk, human colostrum, plasma, urine, and tears, as well as in extracts from certain fetal tissues (S. Kumar, L. Paulesu, M. McKerlie, T. Albrecht, A. Goldman, M. Shabbot, and S. Baron, manuscript in preparation). The occurrence of significant levels of CVI activity in human gastric and duodenal secretions suggests that the antiviral activity may be associated with a stable, acid-resistant molecule. To test this prediction, we investigated the stability of the inhibitor from different sources under acidic and alkaline conditions and after incubation at elevated temperatures. We also investigated the size of the inhibitor, and here we show that the CVI activity is resistant to denaturation by heat, acid, and alkaki and that antiviral activity against a number of viruses resides in a low-molecular-weight molecule.

(This paper was presented in part previously [D. H. Coppenhaver, M. L. McKerlie, S. Kumar, L. Paulesu, and S. Baron, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 429, 1982]).

MATERIALS AND METHODS

Preparation and titration of the inhibitor. CVI was prepared from tissue culture as previously described (1, 4). Other sources of CVI were normal human urine, lyophilized and reconstituted to 5% of its original volume; normal human plasma; powdered bovine milk (Carnation); unpasteurized bovine milk; and normal human gastric, nasal, and salivary secretions. The antiviral activity was titrated in a virus plaque reduction assay as previously described (1, 4). Our standard assay system was carried out on mouse L cells with vaccinia virus as the challenge virus. One unit of CVI activity was defined as the amount of material needed to effect a 50% reduction in plaques formed in the microtiter plaque reduction assay. Activities are given as the reciprocal of the highest titer of test material that gave a 50% inhibition of plaque formation. Statistically significant ($P < 0.05$) differences in CVI titers were threefold.

Viruses. The viruses used in this study were vaccinia virus (strain IHDE) grown in HeLa cells, vesicular stomatitis virus (Indiana strain) grown in chicken embryo cells, herpes simplex virus type 1 grown in rabbit skin cells, and poliovirus type 1 grown in human HEp-2 cells. Stock virus suspensions were frozen in samples at −70°C until used.

Stability tests. Samples were tested for resistance to heat denaturation by incubation for 0.5 h in a water bath at 60°C or in gently boiling water (100°C). Samples were capped to prevent evaporative losses. Plasma, which coagulates at 100°C, was either homogenized after heat treatment or diluted (1:10 [vol/vol]) in phosphate-buffered saline before incubation. After incubation, samples were returned to room temperature and assayed.

Resistance to acid and alkali denaturation was determined by raising or lowering the pH of duplicate 0.5-ml samples of CVI-containing material with 6 N NaOH or 6 N HCl, respectively, followed by incubation at the appropriate pH for 2 h at 37°C. Samples of pH-sensitive murine immune interferon were incubated in parallel as positive controls. After incubation, samples were neutralized and assayed for remaining CVI (or interferon) activity.

Molecular weight determination. Molecular size was determined on calibrated 1.0-by-50-cm columns of Bio-Gel P-6 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 50 mM Tris-hydrochloride, pH 7.5. Columns were calibrated with molecular weight markers as follows: blue dextran (2×10^7), glucagon (3,539), bacitracin (1,423), and tryptophan (240) (Sigma Chemical Co., St. Louis, Mo.). UV absorbance of column effluents was monitored continuously at 280 nm (UA-5 monitor; ISCO, Lincoln, Nebr.). Fractions of 1.0 to 1.5 ml were collected; samples from each fraction were analyzed for antiviral activity. Further details of gel filtration are given in the figure legends. Dialysis tubing (3,500-molecular-weight cutoff) was from Spectrum Industries, Los

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TABLE 1. Stability of antiviral activity in CVI-containing secretions at 100°C

Specimen	Virus titer (U/ml)[a]							
	Vaccinia[b]		VS[b]		Herpes simplex[c]		Polio[d]	
	4°C	100°C	4°C	100°C	4°C	100°C	4°C	100°C
Milk, bovine	27	45	ND[e]	ND	32	81	50	50
Plasma, human	150	138	ND	ND	ND	ND	100	27
Saliva, human	3	6	ND	ND	15	14	<3	<3
Urine, human	32	64	6	3	36	66	25	25
Nasal secretion	6	15	15	6	18	3	3	<3
Gastric secretion	6	8	160	50	ND	ND	ND	ND
Intestinal secretion	35	38	ND	ND	ND	ND	ND	ND
Mouse embryo tissue culture fluid	128	128	64	80	ND	ND	172	217
Human embryonic lung tissue culture fluid	20	75	ND	ND	ND	ND	ND	ND

[a] Average of duplicate determinations.
[b] Assayed on mouse L cells. VS, Vesicular stomatitis virus.
[c] Assayed on rabbit skin cells.
[d] Assayed on human HEp-2 cells.
[e] ND, Not done.

Angeles, Calif. All chemicals used were analytical reagent grade or better.

RESULTS

Stability tests. Preliminary heating experiments established that CVI activity found in mouse tissue culture supernatant and bovine milk was stable after incubation for 0.5 h at 60°C (data not shown). We then investigated the resistance of CVI from a number of sources to heat denaturation after incubation at 100°C for 0.5 h. Results are shown in Table 1. In no case was there a significant, systematic loss of inhibitor activity against all viruses tested when heat-treated preparations were compared to controls kept at 4°C. In a few cases (e.g., plasma CVI assayed against poliovirus) a partial loss of inhibitory activity was observed; such losses were of borderline statistical significance ($P > 0.05$), as were apparent increases in antiviral activity recorded for some samples after heating. Losses from incomplete homogenization of clotted samples undoubtedly contributed to the variability seen. The stable inhibitory activity was characterized as CVI on the basis of its broad antiviral activity and lack of cell species specificity. In no case was inhibitory activity against all viruses tested lost after heat treatment, nor was inhibition of a given virus completely destroyed. Overall, CVI appears stable at 100°C. There are apparent differences in activity in CVI from different sources against different viruses (Table 1). Thus, gastric secretion CVI appears very active against vesicular stomatitis virus and minimally active against vaccine virus, whereas the opposite is true for human urine CVI. Similar results have been presented previously (1).

Three sources of material with CVI activity were tested for resistance to inactivation by extremes of pH. Mouse embryo tissue culture CVI, bovine milk CVI, and human plasma CVI all contained significant antiviral activity after incubation at pH 2 or 12. The inhibitory activity of normal human plasma was reduced somewhat at acid pH, but considerable activity remained even after incubation at pH 1. In contrast, the antiviral activity of murine immune interferon was completely destroyed by both acid and alkali incubation. Titers obtained after incubation at pH 7 were unchanged from unincubated controls. No significant variation in activity was noted for mouse tissue culture CVI or bovine milk CVI throughout the tested pH range. To determine whether the antiviral activity was being stabilized by other molecules in the crude fluids tested, we also treated a partially purified bovine milk-derived CVI preparation obtained from gel filtration chromatography (see below). The antiviral activity of this fraction was stable throughout the range tested.

Gel filtration chromatography. In attempting to concentrate the antiviral activity found in physiological fluids by dialysis against high-molecular-weight solids (Aquacide II; Calbiochem, La Jolla, Calif.), we noted that CVI activity was invariably lost, even when retentive (3,500-molecular-weight cutoff) dialysis membranes were employed. CVI preparations were then dialyzed against a small volume (500 ml) of distilled water, which was subsequently collected and lyophilized. In this case, antiviral activity was found in the lyophilizate, with little or no activity remaining in the retained material. This suggested that the CVI activity was associated with a low-molecular-weight molecule. The size of the inhibitory species was determined by gel filtration chromatography. Mouse embryo tissue culture CVI, human urine CVI, and bovine milk CVI preparations were compared (Fig. 1, 2, and 3). In all cases the major peak of inhibitory activity corresponded to an apparent molecular mass of 2,500 ± 300 daltons. When CVI activity against more than one virus was determined for the same column fraction (Fig. 2, vaccinia virus and herpes simplex type 1 virus), inhibitory activity was found in the same fractions. We assayed various gel filtration column eluants for antiviral activity against vaccinia virus, herpes simplex type 1 virus (Fig. 1, 2, and 3), vesicular stomatitis virus, and poliovirus (data not shown). In all cases, the CVI activity eluted in the same relative position in the column profile. We determined that the antiviral activity was attributable to CVI in each case by characterizing the peaks with respect to the defining properties of CVI (1, 4), i.e., prevention of viral attachment, lack of animal cell species specificity, reversible inhibitory activity, failure to act through cell activation, and failure to be inactivated by incubation at 100°C for 30 min.

DISCUSSION

In earlier reports we have described the presence of an inhibitor of viral attachment found in cell culture supernatants with characteristics which seemed to distinguish it

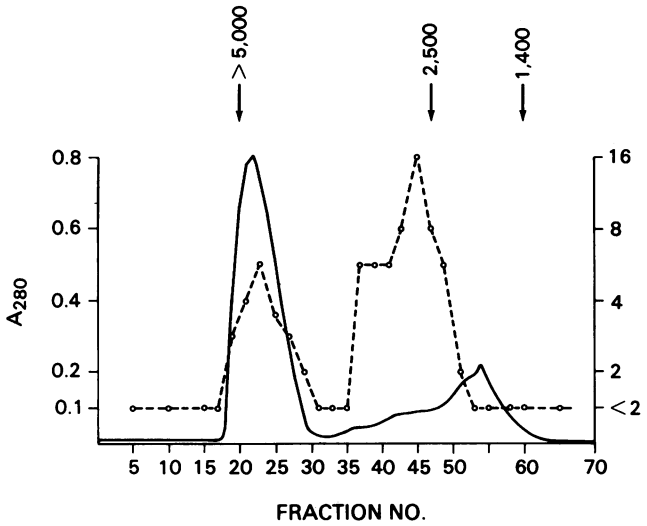

FIG. 1. Molecular size of CVI in mouse embryo tissue culture fluid. A 6.0-ml amount of mouse embryo cell culture supernatant was lyophilized and reconstituted to 10% of its original volume in 50 mM Tris-hydrochloride, pH 7.5. The sample was applied to a 1.0-by-50-cm column of Bio-Gel P-6 and developed in the same buffer at 0.35 ml/min per cm^2. Fractions of 1.4 ml were collected. Total UV absorbance (280 nm, left ordinate, solid line) and antiviral activity against vaccinia virus determined on mouse L cells (right ordinate, broken line) are shown.

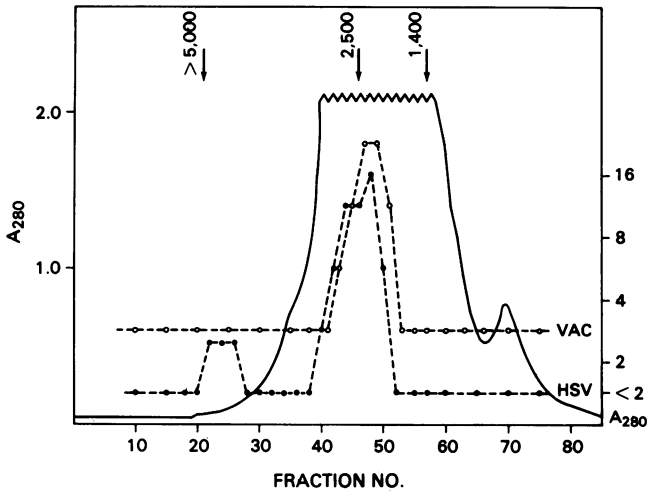

FIG. 2. Coelution of CVI activity against herpes simplex and vaccinia viruses. A 1.0-ml amount of 20-times-concentrated human urine was chromatographed as described in Fig. 1. Fractions of 1.3 ml were collected. Antiviral titers (U/ml) against vaccinia virus (VAC), determined on mouse L cells, and herpes simplex virus type 1 (HSV), assayed on rabbit skin cells, are shown (right ordinate, broken lines). A_{280}, Absorbance at 280 nm (solid line).

from other known viral inhibitors (1, 4). The inhibitor was provisionally identified as a protein or peptide produced by the cells since its appearance was blocked by stopping ribonucleic acid or protein synthesis (1) and the inhibitory activity was not blocked under conditions which would degrade lipids, carbohydrates, and nucleic acids (4). Subsequently, we found an analogous antiviral activity in a number of physiological fluids and tissue extracts (Kumar et al., in preparation). The occurrence of CVI activity in gastric and duodenal secretions enabled us to predict that the inhibitory molecule was acid stable. We were able to confirm this prediction in the present report and to determine that CVI is resistant to denaturation at elevated as well as decreased pH. The finding that partially purified CVI preparations retained their stability to treatment with acid and base gives preliminary evidence that the stability is intrinsic to the inhibitory molecule and not the result of stabilization by other molecular species present in the crude preparations. We have also shown that CVI resists denaturation at elevated temperatures. Recovery of inhibitory activity from lyophilized preparations (Fig. 1, 2, and 3) further demonstrates the stability of the antiviral moiety. Thus, we have determined that CVI is an extremely stable inhibitor which resists inactivation by heat, acid, and alkali.

Gel filtration chromatography experiments (Fig. 1, 2, and 3) show that the inhibitory activity from a number of CVI sources resides in a low-molecular-weight (2,500) molecule. The single exception to this generalization is the CVI activity found in human plasma. Under the same chromatographic conditions as those shown in Fig. 1, 2, and 3, no inhibitory activity was found in the included volume (data not shown). Thus, the CVI activity found in human plasma has an apparent molecular mass of ≥5,000 daltons and is distinguishable from the CVI activity in all other sources examined to date (e.g., mouse embryo tissue culture supernatants, human urine, and bovine milk). It is not clear whether the CVI-like activity found in plasma is then attributable to an entirely different molecule or whether the inhibitory species exists in a different molecular form in the blood. It is possible that the plasma CVI is bound to a specific or nonspecific carrier protein in the blood. Alternatively, it is possible that the inhibitor exists in a polymeric form in the blood and as disassociated monomers in the other sources examined here. Our current data do not allow us to distinguish between these possibilities.

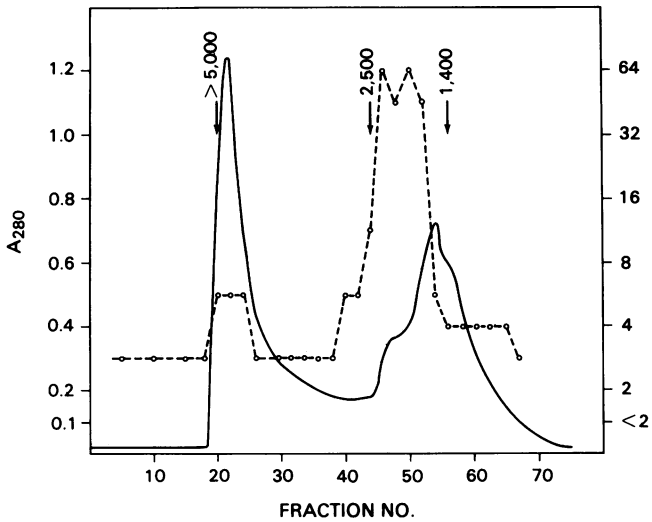

FIG. 3. Molecular size of CVI in bovine milk. Instant nonfat dried milk (Carnation) was prepared in distilled water (50% [wt/vol]), boiled (100°C, 30 min), and centrifuged. The supernatant was lyophilized and reconstituted in column buffer as in Fig. 1. A 0.75-ml amount of sample was applied to the column; 1.5-ml fractions were collected. Other details are as described in the legend to Fig. 1.

The presence of a secondary peak of inhibitory activity was noted in gel filtration experiments with CVI from human urine, bovine milk, and mouse tissue culture supernatants. In all cases the secondary peak of activity corresponded to an apparent molecular weight of ≥5,000. Although these secondary peaks have not been fully characterized as CVI, they do share a number of properties with authentic CVI, i.e., broad antiviral activity and lack of animal cell species specificity. Thus, we tentatively attribute these secondary activity peaks to the same inhibitory species which is found in plasma. The mouse cell cultures were grown in media containing 2% fetal calf serum. It is well known that urine contains low concentrations of serum proteins and that low levels of serum proteins are found in milk. Hence, it is not surprising that some antiviral activity with chromatographic behavior analogous to that observed for plasma CVI was seen in these preparations.

It is known that some relatively low-molecular-weight polyanions are capable of inhibiting virus attachment to host cells. Takemoto and colleagues (8, 9, 12–14) have described the inhibition of encephalomyocarditis, herpes, and influenza viruses by a sulfated polysaccharide derived from agar. The effects of this agar polysaccharide were mimicked by synthetic sulfated polysaccharides such as dextran sulfate (14). It might at first seem that CVI could be related to these polyanions. At least two lines of evidence weigh against this possibility, however. First, dextran sulfate showed significant loss of activity as the molecular weight of the polymer was decreased; activity against poliovirus was lost at a molecular weight two orders of magnitude larger than that determined here for CVI (14), which is active against poliovirus (Table 1). Second, the action of the agar polysaccharide was blocked by the addition of a competing polycation, DEAE-dextran (8). Contrastingly, we were unable to show any significant interaction of CVI with either polycations or polyanions (data not shown). In addition, although we were able to detect a slight plaque inhibitory effect of high-molecular-weight (5×10^6) dextran sulfate in our system, 5,000-dalton dextran sulfate, similar in size to CVI, showed no activity in our assay system, which supports the results reported by Takemoto and Spicer (14). Thus, it seems extremely unlikely that CVI is related to the high-molecular-weight polyanions such as agar-derived sulfated polysaccharide.

The properties of this naturally occurring virus inhibitor which we have here and previously reported continue to delineate CVI from previously described virus inhibitors (2, 5, 11, 15–17). One virus inhibitor with very similar properties was described in human and bovine milk by Matthews et al. (10). Although the properties of the inhibitor described in that report are very similar to those attributed to CVI, CVI seems distinguishable in at least two respects. First, Matthews et al. (10) reported that inhibitory activity is reduced or absent in dried milk preparations. Second, inhibitory activity was destroyed by treatment at 100°C for 30 min. Thus, on the basis of these two criteria, we tentatively conclude that distinct virus inhibitors, or two different forms of the same inhibitor, exist in milk. To avoid possible confusion, we now routinely heat treat CVI preparations (100°C, 30 min) before further characterization. The criterion of heat stability also distinguishes CVI from a newly described bovine leukemia virus-blocking factor found in bovine plasma (3). Similarly, resistance to denaturation by alkali and heat distinguishes CVI from the non-immunoglobulin xenotropic virus neutralizing factor from mouse serum described by Levy and colleagues (6, 7).

The properties of CVI which have been delineated to date include: (i) broad antiviral activity, (ii) lack of animal cell species specificity, (iii) presence in a broad variety of physiological secretions, (iv) resistance to denaturation by heat, acid, and alkali, and (v) low molecular size. The finding that inhibitory activity from different sources and against different viruses is of similar molecular size suggests that a single substance or family of substances is responsible for the antiviral activity in the fluids investigated. The broad activity, potent action, and distribution of this antiviral substance suggest a role in the natural defense of the organism against viral infection.

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