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DIFFERENTIAL INACTIVATION AND SEPARATION OF HOMOLOGOUS AND HETEROLOGOUS ANTIVIRAL ACTIVITY OF HUMAN LEUKOCYTE INTERFERON BY A PROTEOLYTIC ENZYME

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SUMMARY

Human leukocyte interferon (HuLeIF) can express its antiviral activity on both human and bovine cells. The rates of inactivation of HuLeIF by α-chymotrypsin, as expressed on human and bovine cells, are not the same: the ability to induce activity on human cells is lost significantly more rapidly than the activity detected on bovine cells; usually a margin of greater than one hundred-fold exists after α-chymotrypsin treatment. HuLeIF, when subjected to analysis on 10% SDS-PAGE, can be separated into two molecular weight species, one having apparent molecular weight of approximately 21,000 daltons, the other 18,000 daltons. A more rapidly migrating form (molecular weight 16,500 daltons) can also be isolated, which is considerably more active on bovine cells than on human cells. α-chymotrypsin-treated samples analyzed by SDS-PAGE show a clear separation of the activities expressed on human and bovine cells. The residual activity detected on human cells is isolated only in the 21,000 component while the activity found on bovine cells is recovered only as the 16,500 dalton species.

INTRODUCTION

Human leukocyte interferon (HuLeIF) can exert its antiviral activity on a variety of heterologous cells. Among these, the most interesting are rabbit (1), bovine (2), and cat cells (3), which are protected as well as, if not better than, human cells. When HuLeIF is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4), the gel isolates assayed on either bovine cells (5), cat cells (3), or rabbit cells (6), are found to possess both approximately 21,000 dalton and 15,000 dalton forms detected on human cells. Furthermore, their respective activities are nearly equivalent to that on human cells. A faster migrating species (approximately 13,500 daltons) exists, however, which contains as much as 100-times more activity on bovine cells (5) and cat cells (6) than on human cells. It has not yet been ascertained whether HuLeIF ability to display these heterologous activities resides within one

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molecule containing multiple-active sites (7), or distinct subpopulations. One avenue which might resolve these possibilities is to attempt altering HuLeIF in such a manner as to completely separate the activities displayed on homologous and heterologous cells.

Here we report that the antiviral activity of HuLeIF, assayed on human cells, is lost when treated with α-chymotrypsin, whereas the activity displayed on bovine cells is retained. Furthermore, when characterized by SDS-PAGE, the residual activity assayed on human cells is isolated predominantly as the 21,000 dalton species, while the residual activity found on bovine cells is of the 16,500 dalton form.

MATERIALS AND METHODS

Interferons

Human leukocyte interferon (HuLeIF) was produced at the Sloan-Kettering Interferon Production Laboratory, the Theodor Kocher Institute (Berne, Switzerland) by Dr. V.G. Edy, employing a modification of the method previously described by Cantell et al. (8). The preparation employed in the investigation had a specific activity at 4.6×10^5 units/mg.

Interferon samples were assayed on human cells trisomic for chromosome 21 (GM-276; Mammalian Genetic Mutant Cell Repository, Camden, N.J.) and on bovine kidney MDBK cells (generously provided by Dr. P. Sehgal, The Rockefeller University) in a microtitration assay against vesicular stomatitis virus. In this assay, 1 unit was equivalent to approximately 1 NIH HuLeIF unit (reference number G-023-901-527) when assayed on human cells, and approximately 3 units on bovine cells.

α-chymotrypsin treatment

Five mls of HuLeIF containing 8.8×10^5 units of activity, and 1.92 mg protein were dialyzed against 10 mM sodium phosphate buffer, pH 7.6, and loaded onto a Radiometer microtitration vessel (Radiometer, Copenhagen, Denmark). After equilibrating the sample to 4°C, 0.192 mg of α-chymotrypsin (measured spectrophotometrically 1 mg/ml α-chymotrypsin = 0.49 A_{280}; Worthington Enzymes,

Freehold, New Jersey) in 0.1 ml 1mN HCl, pH 3.0, was added. The pH was maintained at 7.6 with a Radiometer pH-stat (Radiometer, Copenhagen, Denmark) and 0.1 N NaOH as the titrant. The temperature was maintained at 4°C with a Haake F2-C circulating water bath (Haake, Saddle Brook, N.J.). Samples (0.1 ml) were removed at 15 min. intervals and the α-chymotrypsin was inactivated with lima bean trypsin inhibitor (Worthington Enzymes, Freehold, N.J.) at an enzyme-inhibitor ratio of at least 1:10.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Treated and untreated HuLeIF samples were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by a modification of the technique described by Stewart II (4). To 0.1 ml samples was added 10 µl of a 10% solution of SDS (final concentration 1%) and 10 µl solution containing 60% sucrose (final concentration 6%) and 0.2% bromophenol blue (final concentration 0.02%). Samples were heated to 100°C for 1 min. and, after cooling, loaded onto 23 cm glass tubes containing 10% polyacrylamide gels. After the dye marker had migrated at least 20 cm, the gels were sliced into 2.2 mm segments and eluted overnight, at 4°C, into 1 ml minimal essential medium containing 10% fetal bovine serum.

RESULTS

α-chymotrypsin treatment of HuLeIF

As depicted in Figure 1, the activity of HuLeIF as detected on human cells was rapidly lost during treatment with α-chymotrypsin while the activity measurable on bovine cells was retained. After 390 min., α-chymotrypsin had reduced the active HuLeIF, assayed on human cells, by more than 99.4%, while the activity discerned on bovine cells remained essentially unchanged.

Interestingly, the inactivation of HuLeIF, assayed on homologous cells, occurred in four distinct steps. The first inactivation began upon addition of the enzyme and the activity then stabilized for approximately 105 min. The second, third and fourth lasted for 90 min., 120 min. and at least 135 min., respectively.

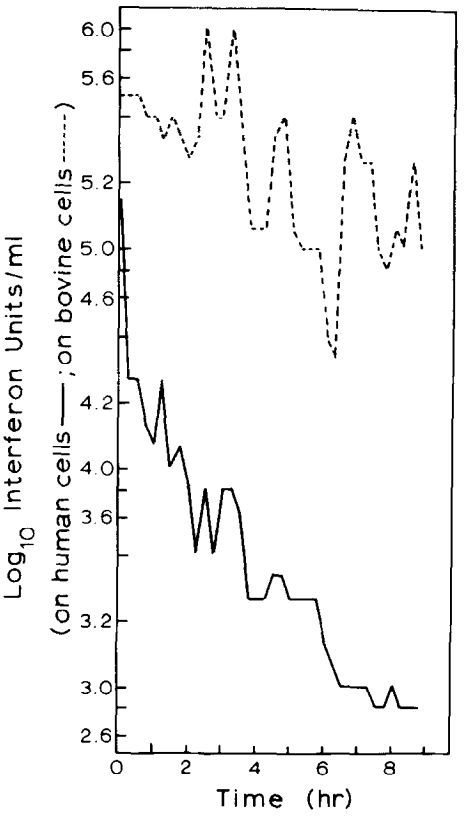
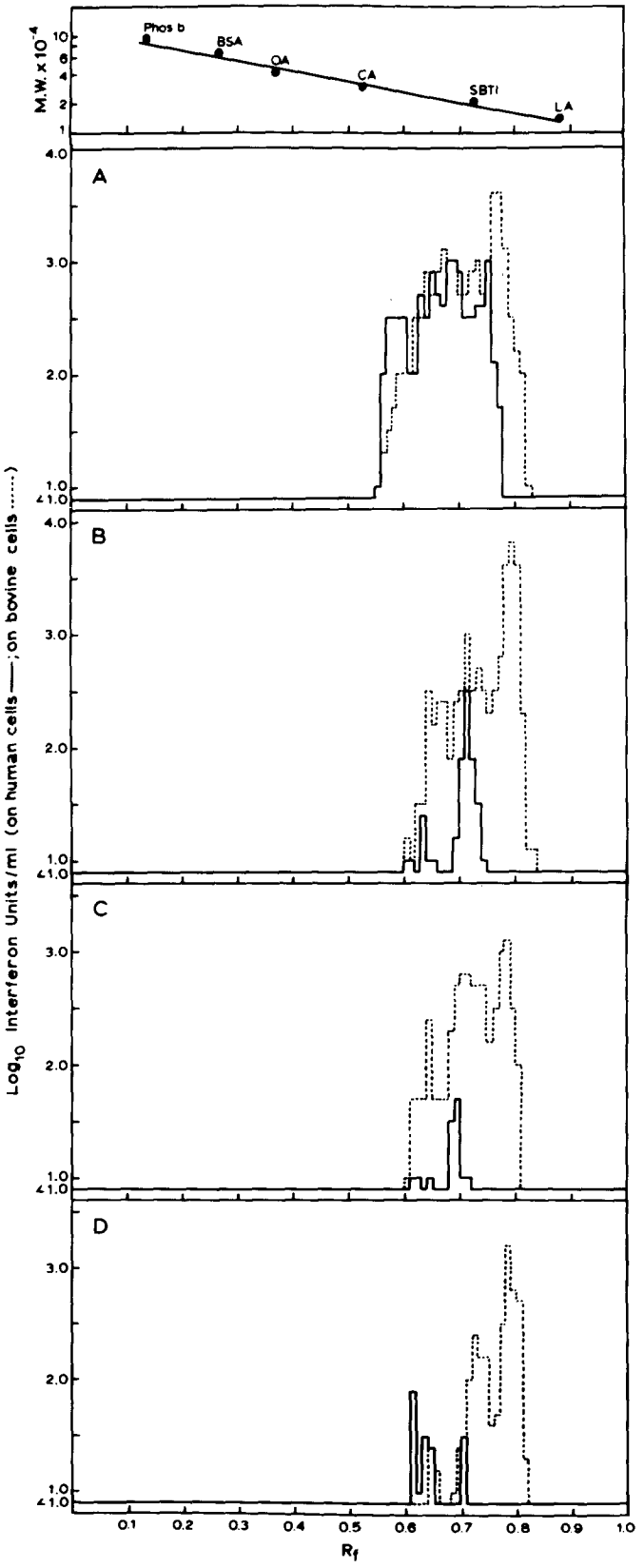


Figure 1. α-chymotrypsin treatment of human leukocyte interferon. A partially purified preparation of HuLeIF was treated with α-chymotrypsin at 4°C and at an enzyme-substrate ratio of 1:10. Samples were removed from the reaction vessel at 15 min. intervals, and the reaction terminated with lima bean trypsin inhibitor at an enzyme-inhibitor ratio of at least 1:10. Samples were then assayed for residual activity on both human and bovine cells.

Separation of residual activities of HuLeIF on homologous and heterologous cells by SDS-PAGE

Homologous and heterologous activities of HuLeIF can be partially separated by electrophoresis in the single dimension (3). Pre-treating HuLeIF, under controlled conditions, with α-chymotrypsin appears further to resolve these two activities.

Figure 2 shows the development at this separation. Panel A represents untreated material. It is noteworthy that the leading edge of the run (i.e., the 16,500 dalton species) contains fractions which have significantly more


Phos b
BSA
OA
CA
SBTI
LA
M.W. x 10⁻⁴
10
6
4
2
1
A
B
C
D
4.0
3.0
2.0
1.0
41.0
Log10 Interferon Units/ml (on human cells ——; on bovine cells .....)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf

activity on bovine cells than on human cells. Panels B, C, and D represent α-chymotrypsin-treated samples harvested from the digestion mixture at 105 min., 285 min., and 435 min. into the reaction. Clearly some of the fractions containing the higher molecular weight form have activity on human cells but not on bovine cells (Panel D, figure 2); conversely, the fractions containing the lower molecular weight species possess activity on bovine cells but not on human cells. The results shown in Figure 2 also show that the progression of the residual activity of treated HuLeIF samples, detected on homologous cells, appears as a 21,000 dalton species, while the activity displayed on heterologous cells is recovered as a 16,500 dalton component.

Similar HuLeIF preparations treated with another proteolytic enzyme, trypsin, resulted in a parallel loss of both human and bovine activities. In addition, when analyzed by SDS-PAGE, the residual activities from these tryptic digests were isolated only as the 18,000 dalton species, when assayed on human cells and as the 16,500 dalton species, when detected on bovine cells (data not shown).

DISCUSSION

In this report we have demonstrated that α-chymotrypsin will readily destroy the antiviral activity of HuLeIF, as assayed on human cells, but leaves the activity displayed on bovine cells relatively undiminished. Thus a means is now available for preparing HuLeIF samples which contain activity only on the heterologous bovine cells.

When the α-chymotrypsin-treated samples are analyzed by SDS-PAGE, the residual activity assayed on human cells was isolated predominantly as a 21,000 dalton species, while the residual activity demonstrated on bovine cells was a

Figure 2. Separation of residual activities of human leukocyte interferon on homologous and heterologous cells by SDS-PAGE. Untreated (Panel A) and treated samples withdrawn from the reaction vessel (illustrated in Fig. 1) at 105 min. (Panel B), 285 min. (Panel C) and 435 min. (Panel D) were analyzed by SDS-PAGE. The eluted fractions were assayed on human and bovine cells. The molecular markers are phosphorylase b (Phos. b), bovine serum albumin (BSA), ovalbumin (OA), carbonic anhydrase (CA), soybean trypsin inhibitor (SBTI), and α-lactalbumin (LA).

16,500 dalton species. Unfortunately, from the data shown (figure 2), the fate of the 18,000 dalton component, when assayed on human cells and the 21,000 dalton component assayed on bovine cells remains unclear. In the case of the former, perhaps the ingredient necessary to convey activity on human cells was more readily lost from the 18,000 dalton species. Concerning the latter, either the 21,000 dalton form was inactivated, or it was converted to the 16,500 dalton entity.

Selecting α-chymotrypsin proved to be quite fortuitous. Since this enzyme cleaves predominantly the carboxyl side of tryptophyl, phenylananyl, and tyrosyl residues of polypeptides, and our HuLeIF preparations contain few of these residues (unpublished data), the number of potential cleavage sites was limited. HuLeIF treated with trypsin resulted in detection of residual homologous and heterologous activities only as 18,000 dalton and 16,500 dalton species, respectively.

The evidence presented does not distinguish between the multiple site (7) and subpopulation hypothesis for species-specificity. However, considering the possible role(s) the carbohydrate moieties could play in regulating species specificity (9), it is tempting to speculate what alteration(s) must occur to maintain heterologous but not homologous activities. If, for example, more, or possibly all, of the attached sugars are required for activity on human, and not bovine cells, it is conceivable that α-chymotrypsin either cleaved or altered that region of HuLeIF containing the glycopeptide bonds, and thus rendered it active only on heterologous cells. Alternatively, the activity displayed on bovine cells could represent a subpopulation which remains stable after α-chymotrypsin treatment.

It is also of interest that, unlike that of mouse interferon produced in the presence of glycosylation inhibitors (10), the molecular weight of HuLeIF was not reduced either by periodate-treatment (11), or when produced in the presence of glycosylation inhibitors (12), or now, after treatment with α-chymotrypsin. This comparison may reflect the extent of glycosylation

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