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Inhibition of the Growth of Human Coronavirus 229E by Leupeptin

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SUMMARY

The protease inhibitor leupeptin prevented multiplication of the human coronavirus strain 229E in cultures of MRC-C cells. The IC_{50} of leupeptin in plaque reduction tests was 0·4 μg/ml, whereas growth of host cells was unaffected by leupeptin at 50 μg/ml. Inhibition of plaque formation could be prevented by the addition of proteases to the overlay medium. In single-cycle growth experiments, leupeptin reduced virus yield only if added within 2 h of infection, indicating its action on an early stage of virus replication.

Proteolytic processing of viral proteins is an essential stage in the growth of viruses belonging to many different families. Although there is no direct evidence for the involvement of proteases in the replication of coronaviruses, recent reports have described the ability of trypsin to stimulate the growth of some coronaviruses in cell culture. For example, trypsin facilitates plaque formation by numerous strains of avian infectious bronchitis virus in chick embryo cell cultures (Otsuki & Tsubokura, 1981), by an enteropathogenic bovine coronavirus in foetal bovine thyroid or brain cells (Storz *et al.*, 1981) and by variants of mouse hepatitis virus in cultures of SRCDF1 DBT mouse cells (Yoshikura & Tejima, 1981). Trypsin also greatly increases the yield of neonatal calf diarrhoea coronavirus from bovine embryonic lung cells under liquid medium (Toth, 1982). These findings suggest the involvement of a protease, possibly occurring in suboptimal amounts in some types of cell, in the growth of a variety of coronaviruses. They do not, however, identify the stage(s) of virus replication that may be affected. We have now observed that the protease inhibitor, leupeptin, suppresses replication of the human respiratory coronavirus 229E in cultures of MRC-C cells. The effect appears to be exerted early in the virus growth cycle.

The MRC-C cell line, derived originally from MRC 5 cells, was obtained from Dr A. F. Bradburne. Human coronavirus strain 229E (HCV 229E), provided by Dr S. E. Reed, was grown in cultures of MRC-C cells under Eagle's MEM plus 0·2% bovine serum albumin. For use as inoculum in single-cycle growth experiments, the virus was concentrated about 30-fold by centrifugation. Plaque assays of HCV 229E were performed in monolayers of MRC-C cells in 50 mm Petri dishes. Virus suspensions were diluted in phosphate-buffered saline plus 10% (v/v) tryptose phosphate broth and volumes of 0·1 ml were allowed to adsorb to the cells for 1 h at room temperature. The monolayers were then overlaid with 5 ml 0·6% agarose in Eagle's MEM plus 50 μg/ml DEAE-dextran and 0·2% glucose. Cultures were stained with neutral red and plaques were counted after 4 days at 36 °C in 5% CO_2.

Plaque reduction tests showed that leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal hemisulphate; Sigma) was a potent inhibitor of the growth of HCV 229E in cultures of MRC-C cells. The concentration of leupeptin required for 50% reduction in plaque count (IC_{50}) varied from 0·2 to 0·8 μg/ml; the mean value was 0·4 μg/ml (0·8 μM), and plaque formation was usually suppressed completely by leupeptin at 2 μg/ml. In a single test, HCV 229E was equally susceptible to leupeptin when growing in the L132 line of human embryonic lung cells (IC_{50} 0·3 μg/ml). The morphology of MRC-C cells was unaffected by concentrations of leupeptin up to 25 μg/ml in the agarose overlay, and under liquid medium the cells multiplied normally for at least 3 days in the presence of 50 μg/ml leupeptin.

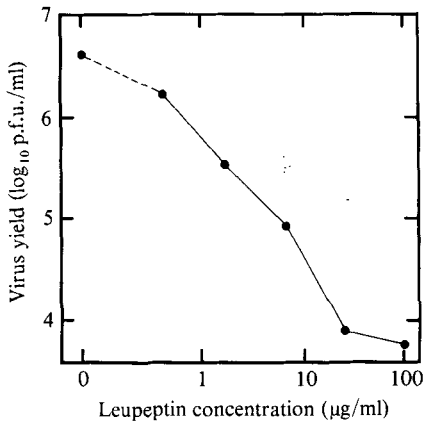

Fig. 1

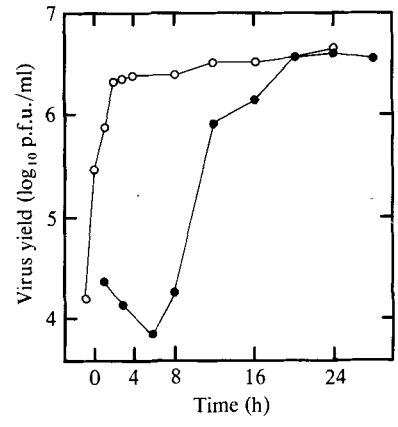

Fig. 2

Fig. 1. Inhibition of growth of HCV 229E by leupeptin. Cultures of MRC-C cells were infected with HCV 229E as described in the text and leupeptin was included in the overlay medium at a range of concentrations. Cultures were harvested after incubation for 24 h.

Fig. 2. Single-cycle growth curve of HCV 229E, and inhibition of growth by leupeptin. Cultures of MRC-C cells were infected with HCV 229E as for Fig. 1. Some cultures (●) were harvested at the times indicated. Other cultures (○) received leupeptin (100 μg/ml) at the times indicated and were harvested at 24 h.

Inhibition of plaque formation by leupeptin could be prevented by the simultaneous incorporation of a protease in the overlay medium. The enzyme most studied was thermolysin (EC 3.4.24.4; Sigma) which at 1 μg/ml increased the IC_{50} of leupeptin to more than 25 μg/ml. Trypsin, subtilisin and Pronase also prevented inhibition by leupeptin but chymotrypsin, elastase, collagenase and bromelain had little or no effect. Plaques formed in the presence of the active proteases were slightly enlarged; they also contained syncytia which were not normally seen, a finding consistent with earlier observations on the effect of trypsin on other coronaviruses (Storz *et al.*, 1981; Yoshikura & Tejima, 1981; Toth, 1982).

To investigate the effect of leupeptin on virus yield, MRC-C cultures (about 6×10^5 cells) in 35 mm dishes were infected by adsorption of virus from 0·1 ml concentrated suspension of HCV 229E (6×10^7 p.f.u./ml) for 1 h at 36 °C. The cultures were washed twice and overlaid with 2 ml Eagle's MEM plus 0·2% bovine serum albumin and 20 mM-HEPES buffer pH 7·4 (maintenance medium). Leupeptin at a range of concentrations was added to the virus inoculum, to the maintenance medium and also to maintenance medium used to treat the cultures for 1 h before infection. After 24 h at 36 °C in air, the cells were scraped into the medium and disrupted by sonication. Fig. 1 shows that virus yields, as p.f.u./ml, were reduced by all concentrations of leupeptin from 0·4 to 100 μg/ml, the maximum reduction being 2·8 $\log_{10}$ units.

To determine the time of action of leupeptin, cultures of MRC-C cells were infected with HCV 229E as in the previous experiment and leupeptin at 100 μg/ml was added at times ranging from 1 h before infection to 24 h after infection. The cultures were harvested and assayed for virus at 24 h. Other cultures, without leupeptin, were harvested at intervals to establish the normal growth curve. Fig. 2 shows that, in cultures without inhibitor, new virus was first detected at 8 h and attained maximum titre by 20 h. Leupeptin reduced the final virus yield by 2·3 $\log_{10}$ units if present from 1 h before infection. However, it was appreciably less effective when added only at the time of infection and caused little or no inhibition if added 2 h later.

These results indicated that leupeptin affected an early event in the virus growth cycle. The compound had no direct action on the virus, for virus infectivity was unaffected by incubation with 100 μg/ml leupeptin for 2 h at 36 °C. The effect of leupeptin on the attachment of virus to cells was investigated by including leupeptin in the diluent from which virus was allowed to adsorb to cultures of MRC-C cells. At intervals, the cultures were washed, overlaid with agarose

medium containing 1 μg/ml thermolysin to nullify the action of remaining leupeptin, and incubated to allow the development of plaques. The presence of leupeptin at concentrations up to 100 μg/ml did not affect the amount of virus that became adsorbed.

It seemed unlikely, from its time of action, that leupeptin interfered with the proteolytic cleavage of a virion surface protein, such as that required for the acquisition of infectivity by myxo-, paramyxo- and rotaviruses. However, to test this possibility, HCV 229E was grown in the presence of 100 μg/ml leupeptin, freed from leupeptin by centrifugation and then incubated for 2 h at 36 °C with thermolysin or trypsin at concentrations from 0·1 to 3 μg/ml. This treatment failed to enhance infectivity, and so provided no evidence that leupeptin caused the formation of virus particles with an abnormal, uncleaved surface protein.

Other protease inhibitors were tested for their ability to affect plaque formation by HCV 229E. Soybean, lima bean, chick ovomucoid and turkey egg white inhibitors, pepstatin A at 100 μg/ml and aprotinin at 400 u/ml all failed to inhibit. Antipain inhibited plaque formation with an IC_{50} of 40 μg/ml (60 μM) and, like leupeptin, its effect was prevented by thermolysin. *p*-Nitrophenyl *p'*-guanidinobenzoate also inhibited, the IC_{50} being 75 μg/ml (22 μM). However, this compound was slightly toxic to the cells and its effect could not be prevented by proteases. Hence, the specificity of its action was open to doubt.

Two other coronaviruses were examined for their susceptibility to inhibition by leupeptin. Feline infectious peritonitis virus was titrated in cultures of feline embryo lung cells in microtitre plates. In the presence of leupeptin at 0, 20 and 100 μg/ml, infectivity titres were $10^{5·7}$, $10^{5·7}$ and $10^{5·3}$ ID_{50}/ml respectively, showing that leupeptin had little or no effect on the growth of this virus. Plaque reduction tests with porcine transmissible gastroenteritis virus in cultures of LLC-PK1 cells were carried out by Dr E. A. Rollinson (Wellcome Research Laboratories, Berkhamsted, U.K.); the IC_{50} of leupeptin against two strains of virus was greater than 25 μg/ml.

Our results show that leupeptin can suppress growth of the human coronavirus 229E in MRC-C cells at concentrations well below the cytotoxic level. Antipain, which has a similar but not identical specificity to leupeptin (Aoyagi & Umezawa, 1975), inhibited to a lesser extent. Other protease inhibitors had little or no effect. Leupeptin inhibits certain serine and thiol proteases, e.g. trypsin, plasmin, papain and cathepsin B, which cleave peptide bonds on the carboxyl side of arginine residues (Aoyagi & Umezawa, 1975). Hence, such an enzyme is probably concerned in the replication of HCV 229E. However, a variety of proteases, including thermolysin which does not react with leupeptin (Aoyagi & Umezawa, 1975), was able to nullify the effect of leupeptin. This implies that the protease activity required by the virus is relatively non-specific.

Since leupeptin inhibited most effectively if added 1 h before the virus, the compound may take some time to reach or react with its target enzyme. Hence, 'time of addition' experiments cannot reveal precisely the time of inhibitory action. Nevertheless, it is evident that leupeptin exerted its effect early in the virus growth cycle and probably within 2 h of infection. Leupeptin did not directly inactivate virus infectivity or prevent adsorption of virus to cells, and there was no evidence that it prevented cleavage of a surface protein of newly formed virus particles. Andersen (1983) found that leupeptin inhibits retrovirus infection in mouse fibroblasts, and suggested that inhibition of lysosomal proteases prevents entry of virus into the cytoplasm. The suppression of coronavirus growth may have a similar explanation.

The lack of activity of leupeptin against feline infectious peritonitis and porcine transmissible gastroenteritis viruses was somewhat surprising, particularly as these viruses fall into the same antigenic group as HCV 229E (Siddell *et al.*, 1983). However, the host cells differed from those used for growth of HCV 229E. If, as seems likely, leupeptin acts on a cellular enzyme, then the nature of the host cell could influence susceptibility to inhibition.

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