Antiviral Activity of Tripeptides against Respiratory Syncytial Virus

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(Recieved on 17. August, 1994)

Oligopepetides with amino acid sequence similar to those at the N-termini of the F_1 viral polypeptides were examined for their inhibitory effect on respiratory syncytial (RS) virus plaque formation in Hela cells. Various protecting groups at the N-termini of tripeptide (Phe-Leu-Gly) were used for the inhibition against RS virus.

The presence of benzyloxycarbonyl group (Z) on the N-terminal amino acid of the tripeptides increased the inhibitory activity, and the tripeptide containing $_D$-phenylalanine decreased the activity.

Introduction

Most frequent among the causative agents of acute respiratory infection in infants are respiratory syncytial (RS) virus (1-4). RS virus is the most important cause of lower respiratory tract infections in infants younger than 12 months. Approximately half of all infants become infected with RS virus in the first year of life and the remainder in their second year (5-7). Sometimes the disease is severe enough to require hospitalization. Mortality due to RS virus infection has been estimated as 0.5% in infected children but this may rise to 23% in immunocompromised infants and 37% in those with congenital heart failure (8-10). Ribavirin has been licensed for clinical use in the treatment of severe RS virus infections. However, ribavirin is not widely used as a treatment for myxovirus infections, particularly because the aerosolized compound has to be administered in a hospital setting. Moreover, ribavirin is not highly selective as an antiviral agent, in that it also influences host cell metabolism (11, 12). A formalin-inactivated RS virus vaccine is not protected from natural infection with this virus during subsequent epidemics and develop more severe pneumenia than children without prior vaccination (13, 14). Recent research efforts have focused on the development of effective antiviral compounds.

Oligopeptides, which have amino acid sequences similar to those of the N-terminal regions of paramyxovirus F_1 polypeptide or the myxovirus HA_2 polypeptide, are highly active, specific inhibitors of the infectivity of each virus, and of cell fusion and hemolysis induced by paramyxoviruses (15).

This has prompted us to search for antiviral oligopeptides against RS virus which has general similarities with the paramyxo-viruses and is classified provisionally within the family. As with other paramyxoviruses, the envelope of RS virus contains two surface glycoprotein. The M_r 84,000 glycoprotein (G) mediates attachment to target cells and the second, M_r 68,000 glyco-

Scheme I

Virus	F1 Peptide
Sendai	FFGAVIGTIALGVATSAQIT
Influenza (A)	GLFGAIAGFIEGGWTGMIDG
Influenza (B)	GFFGAIAGFLEGGWEGMIAG
RSV	FLGFLLFVGSAIASGVAVSK

Antiviral Activity

Z-$_D$F-$_D$F-G	EC_{50} 10 mM	Sendai Virus
Z-$_L$F-$_L$F-G	EC_{50} 23 mM	Sendai Virus
Z-G-$_L$L-$_L$F-G	EC_{50} 20 mM	Influenza (A) Virus
Z-G-$_L$F-$_L$F-G	EC_{50} 53 mM	Influenza (B) Virus

(Z = Benzyloxycarbonyl)

[C.D. Richardson et al., *Viology*, **105**, 205-222 (1980).]

protein is the viral fusion (F) glycoprotein. The active paramyxovirus F protein consists of two disulfide-linked units, F_1 and F_2, which are generated from an inactive precursor, F_0, by a specific internal cleavage by cellular protease. The availability of an appropriate cellular protease and the suceptibility of F protein to the requisite cleavage are important parameters in paramyxovirus pathogenesis. Collins et al. clarlified the sequence of F protein, of which F_1 protein is strongly hydrophobic and consists in 20 hydrophobic amino acids (16). We tried to use tripetide (Phe-Leu-Gly) of N-terminal part of this F_1 protein.

Materials and Methods

Cells and Virus.

Two strains of RS virus (subtype A) were used: the Long strain (standard strain) was provided by Dr. Y. Numazaki (Sendai National Hospital) and the FM-58-8 strain isolated from an infant with bronchopneumonia at the Paediatric Clinic of Fukusima Medical College Hospital. Both strains were passed several times in HEp-2 cells in our Laboratory.

HeLa cells were cultured in Eagle's essential medium (MEM) supplemented with 10% newborn calf serum (NCS), 1.6% glucose (16 folds higher concentration than in ordinary MEM), 100 units ml^{-1} of penicillin-G and 100 $\mu g\ ml^{-1}$ of streptomycin. For the RS virus assays in Hela cells, the maintenance medium consisted of concentration of glutamin, 1.6% glucose and the antibiotics mentioned above. Vero cells were cultured in MEM plus 10% NCS and antibiotics.

Antiviral assays

The cells were seeded in 24-well tissue culture plates (Falcon 3407, Becton Dickinson Co., Oxnard, CA, USA) at 1.5x 10^5 cells per well and incubated at 37 °C. After a 1-to 2- day incubation period, when the cell cultured had reached confluency, the growth medium was withdrawn and the cell monolayers were washed once with MEM. To each well were added 20 to 40 plaque forming units (PFU) of virus in 0.1 ml maintenance medium. Simultaneously with the virus 0. 2 ml of serial 4-fold dilutions of peptides in maintenance medium and 0.7 ml of 1% methyl cellulose (Methpcel A-4M Premium, Dow Chemical Co., Midland, MI, USA) in maintenance medium were added to the wells. Three wells with a monolayer each were each used for one dilution of peptides. The infected cell cultures were incubated at 35°C in a CO_2 incubator. After a 3- to 4-day incubation period, the overlay medium was withdrawn and the cell sheet was stained with 0.1% neutral red solution for 1 h at 37°C. After staining, the monolayers were fixed with 5% formalin and the number of plaque was determined under the microscope (40x magnification). An average number of plaques was calculated and peptide concentration required to inhibit the number of plaques or haemadsorbing foci to 50% of the control value was estimated as the 50% effective concentration (EC_{50}).

Inhibitory effects of peptides on viral antigen synthesis and syncytium formation were monitored by counting the numbers of antigen positive foci or cells in a syncytium after immunofluorescent staining of the infected cells. HeLa and Vero cells were seeded in Lab-Tek chamber slide (8. chambers, Nunc Inc., Naperville, IL, USA) and incubated at 37°C in 5% CO_2. When the cell sheets had become confluent, approximately 50 PFU of RS virus was inoculated in each chamber (Hera and Vero cells, respectively). Serial 4-fold dilutions of peptides in maintenance medium with 0.7% methyl cellulose were added to the chambers (in triplicate) and the cultured were incubated at 37°C in a CO_2 incubator. At 36 h after infectios, the maintenance medium was withdrawn, cells were washed twice with phosphate buffered saline (PBS, pH 7.2) and fixed with acetone for 10 min at room temperature. The fixed cells were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit antibodies against RS virus (Denka Seiken Co., Niigata, Japan) for 30 min at 37°C, After staining, the cells

were washed with PBS and mounted with 20% glycerol in PBS. Immunofluorescence was analysed under a fluorescent microscope (Nikon Optiphot+EFD2, Nikon Industrial Co., Tokyo, Japan).

Compounds

Peptides were synthesized by the classical solution method. The synthesis of peptides in solution of methylene chloride involved coupling the benzyloxycarbonyl amino acids with methyl or ethyl esters of other amino acids or peptides which possesed a free amino terminus. Dicyclohexylcarbodiimide was employed as a coupling reagent. In this case, hydroxybenzotriazole was used as an activating agent. N-Benzyloxycarbonyl-peptide esters were saponified with 0.5 N NaOH aqueous solution to remove the ester moiety. Other N-protecting groups were induced by means of an usual manner after removal of the benzyloxycarbonyl group of peptide esters by catalytic hydrogenation.

Cytotoxic Measurement

The cytotoxicity of the peptides was examined by the trypan blue exclusion test and inhibition of RNA or DNA synthesis. For the former test, HeLa cell cultures were prepared in plastic trays containing 24 wells (16-mm diameter ; Falcon Plastics). After 2 days of incubation at 37°C in a CO_2 incubator, when the cell cultures were confluent, culture medium was removed from each well and 1-ml volume of maintenance medium containing serial concentrations of the test peptides were added.. For the cell control, 1 ml of maintenance medium without the peptide was added. All cultures were incubated at 37°C, and after 2 and 7 days of incubation, peptides were withdrawn and the viability of the cells was determined by the trypan blue exclusion method. The concentration of peptide that reduced the viability of HeLa cells to 50% of the control was estimated as the 50% cytotoxic dose (CD_{50}).

The inhibition of RNA and DNA synthesis was examined as follows. Approximately $4x10^4$ cells were seeded per well in plastic trays containing 96 wells, and serial concentrations of the peptide were added simulteniously (per three wells). [5-^{3}H]Uridine (specific radioactivity, 29 Ci/mmol) or[methyl-^{3}H]deoxythimidine (specific radioactivity, 25 Ci/mmol) was added at 125 nCi per well at the time of cell seeding. After incubation at 37°C for 16h, the cells were harvested, applied to glass filters, and washed twice with phosphate-buffered saline (pH 7.2), five times with 5% trichloroacetic acid (TCA) and finally five times with 95% ethanol. The TCA-insoluble radioactivity was counted in a liquid scintillation counter, and the concentration of peptide which inhibited the incorporation of uridine or deoxythymidine to 50% of the control value was determined as the 50% inhibitory dose (ID_{50}).

RESULTS

The sequences of the first 20 residues of the F_1 polypeptides of RS virus was reported (17). The highly hydrophobic nature of this region of the polypeptide is evident. Based on the above sequences, oligopeptides were synthesized to resemble the N-terminal region of F_1 peptide of RS virus, but also introducing variations in sequence, steric configuration, and addition of groups to the N-terminal residue. In addition, the tripeptide, Z-$_D$-Phe-$_D$-Phe-$_L$-Leu, which was shown to inhibit the replication of paramyxovirus (18), was also used.

Although these oligopeptides inhibit virus-induced cell fusion and homolysis as well an virus infectivity at the level of penetration, in most experiments inhibition as the assay system was employed, because of the ease and greater sensitivity of this method.

ED_{50} and CD_{50} of oligopeptides for replication were shown at Scheme II.

Z-$_L$-Phe-$_L$-Leu-Gly which mimic the N-terminus of the F_1 poly-peptide were the most effective. On the other hand, optically isomer, Z-$_D$-Phe-$_L$-Leu-Gly was surprisingly no active, and inducing other protecting group instead of

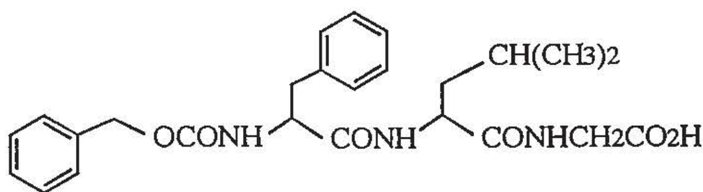

Fig. 1　Z-FLG

Scheme II

Specific Inhibition of RS Virus Replication by Z-Tripeptides

Z-$_L$F-$_L$L-G EC_{50} 2.7 μg/mℓ

(Z-$_D$F-$_L$L-G EC_{50} > 200 μg/mℓ)

Antiviral Activity of Tripeptide (FLG) having other N-Protecting Group against RS Virus.

Phenoxyacetyl-FLG	EC_{50} 42.9 μg/ml
p-Nitrobenyzloxycarbonyl-FLG	EC_{50} 88 μg/ml
3-Phenylpropionyl-FLG	EC_{50} > 200 μg/ml
Mandelyl-FLG	EC_{50} > 200 μg/ml
Phenylacetyl-FLG	EC_{50} > 200 μg/ml
Trifluoroacetyl-FLG	EC_{50} > 200 μg/ml

Z-group eliminated the activity. Other oligopeptides having different amino acid sequences from F_1 polypeptide were also inactive.

These results indicated that the inhibition is amino acid sequence specific. The presence of the correct N-terminal amino acid of a sequence is of prime importance, but even the second amino acid is factor in the level of activity. The stereochemistry of amino acids is important to show activity against RS virus. Tripeptide having the $_L$-form amino acid sequences only inhibits the combination of virus to host cell. It seems that the structure of surface lipidprotein of the host cell is concerning with the combination, and $_L$-form tripeptide blocks the acceptor of surface of host cell to inhibit the combination with RS virus. The precise mechanism of action of these oligopeptides remains to be elucidated ; however, the data suggest that they act by interfering with the function of the N-terminal region of the F_1 polypeptide of RS virus. Indeed, the present results provide evidence in addition to that enumerated in the introduction that the N-terminal regions of these polypeptides are involved in viral penetration, virus-induced hemolysis and cell fusion. We have not carried out detailed studies on possible cytotoxic effects of oligopeptides. However, they do not appear to be significantly toxic in the systems studied.

The present studies have several biological implications. They have provided further evidence for the importance of a specific amino acid sequence at the N-terminus of the F_1 polypeptide of RS virus in the initiation of infection. The finding that the initiation of infection can be inhibited specifically by oligo-peptides synthesized to resemble a region of a viral glycoprotein, provides a possible new approach to chemical inhibition of virus replication.

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