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ISOLATION OF IMMUNIZING CYANOGEN BROMIDE-PEPTIDES OF FOOT-AND-MOUTH DISEASE VIRUS

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SUMMARY: The isolated structural protein with the N-terminal amino acid threonine of foot-and-mouth disease virus, type O_1 strain Kaufbeuren was treated with CNBr and the cleavage peptides were separated by gel filtration on Sephadex G-100 followed by ion exchange chromatography on phosphocellulose. Two peptides with molecular weights of about 5.200 daltons still capable of inducing antibodies in guinea pigs were purified. The antibodies were found to neutralize the homologous foot-and-mouth disease virus as detected by the neutralization test in suckling mice. The findings strongly suggest that the primary structure of small CNBr cleavage peptides carries antigenic determinants similar to those on the native virus protein with the N-terminale amino acid threonine.

Foot-and-mouth disease virus (FMDV) was classified as a member of the rhinovirus group of animal picornaviruses. Its protein coat was found to consist of three major structural proteins arranged in an icosahedral pattern (1, 2), a fourth smaller protein and a trace of the precursor protein (3, 4, 5). The N-terminal amino acids of two structural proteins, glycine and aspartic acid, are identical in all picornaviruses so far investigated (6). The N-terminal group of the third major FMDV structural protein, however, was identified as threonine (VP_{Thr}) (7, 8) whereas other picornaviruses carry serine as the N-terminal group (6). Among them only VP_{Thr} was shown to induce and absorb neutralizing (9) antibodies and to protect swine against challenge infection with virulent FMDV as reported for VP_{Thr} of FMDV, type A_{12} strain (10).

Recently we have reported that CNBr-cleaved peptides of VP_{Thr} of FMDV, type O_1K, induced neutralizing antibodies and partial immunity in guinea pigs (11). Here, we report the separation of two peptides with

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molecular weight of about 5,200 daltons from CNBr-cleaved VP_{Thr} of FMDV O_1K and induction of low but reproducible titres of neutralizing antibodies. The findings of the electrophoretic and gel filtration experiments provide further support for the view that single peptides of denatured VP_{Thr} without detectable amounts of contaminating uncleaved VP_{Thr} are still capable of inducing neutralizing antibodies directed against FMDV.

MATERIAL AND METHODS:

Growth and purification of virus. The experiments were performed with a plaque-purified isolate of FMDV type O_1, strain Kaufbeuren. Virus was grown in roller bottles of BHK 21 cells. After concentration by precipitation with polyethylene glycol the virus was purified by CsCl cushion centrifugation followed by a sucrose density gradient centrifugation (2).

Isolation of capsid protein VP_{Thr}. Capsid protein was isolated from purified 146S-particles of FMDV after RNA precipitation using ion exchange chromatography on CM-cellulose (7, 12).

Cyanogen bromide cleavage of capsid protein VP_{Thr}. The method applied by Schroeder et al. (13) was followed. Twenty mg of isolated VP_{Thr} were dissolved in 1 ml of 70% (v/v) trifluoro acetic acid and were allowed to react with 150 mg CNBr. The mixture was left for 48 h at 20°C in the dark. The CNBr peptides were oxydized using performic acid as described by Hirs (14).

Isolation of CNBr peptides by gel and ionexchange chromatography. For gel filtration Sephadex G-100 (Pharmacia) superfine, particle size 25-32 µ, was equilibrated in 40% (v/v) acetic acid, 10% (v/v) propionic acid in a 1.5 x 95 cm column. Fractions of 570 µl each were isolated at a flow rate of 2.3 ml/h. Protein samples of 10 mg CNBr peptides were applied in 0.5 ml volumes. The effluent from the column was monitored at 280 nm. Peak fractions isolated by gel filtration were subjected to ion-exchange chromatography on phosphocellulose P-11 (Whatman) on a 0,5x7 cm column. The peptides were eluted using a linear gradient formed from 0.01 M sodium formate, pH 2.7, 6 M urea and 0.05 M sodium formate solution, pH 2.7, 0.7 M NaCl, 6 M urea. Fractions of 1 ml each were collected and the peptides were detected at 280 nm. Fractions containing peptides were desalted on Biogel P-2 suspended in 90% formic acid.

Protein determination. 50 µl aliquots of the peptide fractions were dried and hydrolyzed with 125 µl of 0.5 N NaOH for 20 min at 120°C. After cooling in an ice bath, 100 µl of 0.5 N HCl and 250 µl of sodium borate buffer, pH 8.5, with 0.1% (v/v) Triton X-100 were added. Finally, 75 µl 0.03% (w/v) fluorescamine dissolved in isopropanol were added. Other technical details are identical to those described by Nakai et al., (15).

Polyacrylamide gel electrophoresis. The homogeneity of the isolated CNBr peptides was controlled by sodiumdodecylsulfate (SDS)-urea slab gel electrophoresis using the conditions of Swank and Munkres (16). For the preparation of a 5% stacker gel, the acrylamide:bisacrylamide ratio was changed to 20:1. After electrophoresis, the peptide bands were detected by staining with Coomassie brilliant blue. In some experiments, peptides were labelled with Fluram and identified by exposing the gels to UV light of 366 nm. For the fluorescamin labelling Tris in the stacker gel buffer was replaced by NaOH.

Production of antisera. Three doses of 200 µl each (protein content 70 - 100 µg) of (peak) fractions were emulsified with complete Freund's adjuvant and injected into guinea pigs by the intramuscular route in ten-day-intervals. Three animals (body weight 400 g) were used in each group. Two control groups received similar doses of 0.1% SDS dissolved in phosphat buffered saline (PBS) or purified FMDV O_1K 146 S-particles. The first blood samples were taken four weeks after the last antigen application followed by other bleedings in eight-days-intervals. Pre-sera and immune sera were tested in the virus neutralization test with the homologous FMDV and heterologous bovine enterovirus, strain LCR.

Virus neutralization tests. Fivefold dilutions of guinea pig antisera were mixed with equal volumes of 500 LD_{50} (letale dosis 50 percent) of FMDV O_1K and incubated at 37°C for 30 min. Subsequently, 0.1 ml volumes of the mixtures were inoculated intramuscularly into four-days-old mice. The neutralizing activity was determined as the serum dilution protecting 50% of mice. For control experiments with bovine enterovirus about 100 $TCID_{50}$ (tissue culture infections dosis 50 percent) were incubated with the guinea pig antiserum dilutions, incubated at 37°C for 30 min and titrated in roller tubes of MDBK cells.

RESULTS: The CNBr-cleaved VP_{Thr} peptides were separated by gel filtration on a Sephadex G-100 column. For convenient reference, a representative elution profile of isolated VP_{Thr} peptides is illustrated in Figure 1. According to the optical density at 280 nm, fifteen different protein peaks were eluted from the column and were each used for hyperimmunization of guinea pigs. Significant virus-neutralizing activity was detected in guinea pig sera corresponding to the peptide fractions 8 and 9. The protein content as determined by the fluorescamine technique appeared to vary considerably and by the electrophoretic analysis no typical peptide pattern was detected. Figure 2a shows the gel electrophoresis pattern of the fluorescamine-labelled peptide fractions 8, 9 and 10. It appeared that these fractions were electrophoretically heterogenous. Consequently, each fraction was further purified by ion exchange chromatography on phospho-cellulose P-11. The elution profile of the peptide fraction 9 from the phosphocellulose column is represented in Figure 3. The first peak as detected by optical density measurement at 280 nm did not give protein-specific reactions and was therefore considered to be non-specific. The following fractions, however, designated as 9_{1-5} migrated homogenously in the polyacrylamide gel electrophoresis as shown in Figure 2b. Fraction 9_5 appeared to be identical with fraction 8 according to its elution

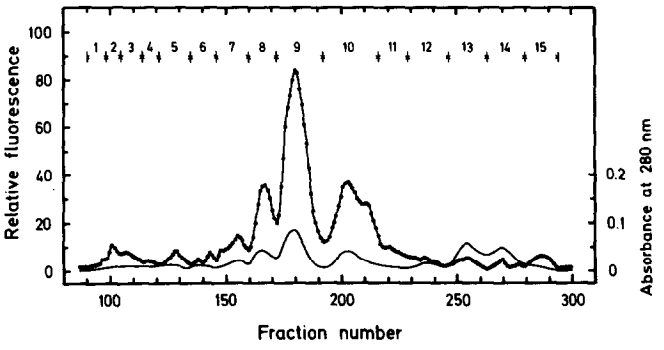

Fig. 1 Separation of VP_{Thr}-CNBr peptides on a column of Sephadex G-100. For further details see Material and Methods. ——— Absorbance at 280 nm, ... Relative fluorescence.

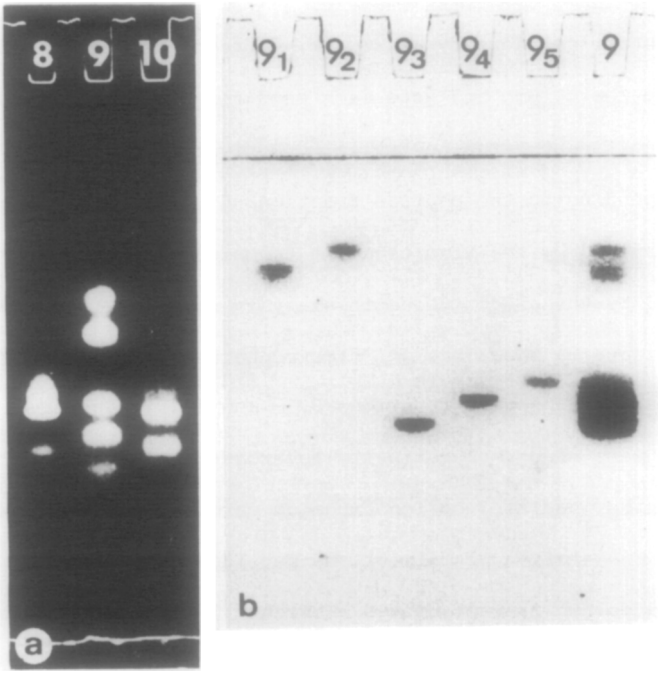

Fig. 2 Polyacrylamid gel electrophoresis of the separated fractions.

a) Fractions 8, 9, 10 of the G-100 separation. The peptides were stained with fluorescamine before the electrophoresis.
b) Fractions 9 after the separation on the column of phosphocellulose P-11, stained with Coomassie blue.

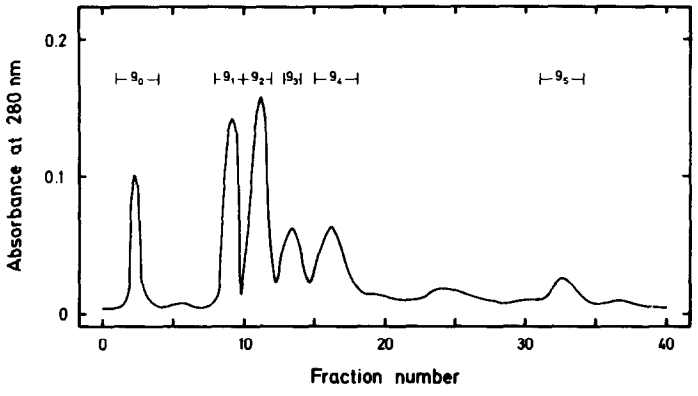

Fig. 3 Purification of CNBr peptide fraction 9 from the Sephadex G-100 column on a column of phosphocellulose P-11. For further details compare Materials and Methods.

characteristics by gel filtration and ion exchange chromatography thus suggesting an incomplete separation of the peptides by gel filtration. The titres of the virus-neutralizing antibodies detected in guinea pig sera are summarized in Table 1. Low but significant antibodies titres were seen in sera raised against fractions 9_3 and 9_1. The minor neutralizing activity of the serum produced against fraction 9_4 was found due to an incomplete separation of the peptide 9_3.

DISCUSSION: The findings presented in this study confirm and extend a previous communication about the CNBr cleavage product of VP_{Thr} of foot-and-mouth disease virus O_1K. It was reported that the CNBr peptides were capable of inducing significant titres of virus-neutralizing antibodies and at least partial immunity against challenge infection in guinea pigs (11). The immune response in the animals immunized with the CNBr peptides of FMDV strain O_1K was weaker than that of the 146S particles. It has to be mentioned, however, that the small peptides used in this study were administered without carrier proteins only supplemented by complete Freund's adjuvant. The detection of virus-neutralizing antibodies induced by two isolated peptides with mol.wt. of 5.200 clearly demonstrated that

Table 1: Titres of FMDV-neutralizing antibodies in guinea pigs immunized with fractions of isolated VP_{Thr}-CNBr peptides of FMDV O_1K.

Antiserum prepared against	Reciprocal serum dilution (ND_{50}/0,1 ml)		
	4 weeks p.i.	5 weeks p.i.	6 weeks p.i.
G-100 separation			
Fraction 8			90
Fraction 9			120
PBS with 0.1%SDS			-
FMDV O_1K, 146 S			480
P-11 separation			
Fraction 8	-	-	-
Fraction 9_1	-	5	35
Fraction 9_2	-	-	-
Fraction 9_3	-	15	45
Fraction 9_4	-	-	10
PBS with 0.1%SDS	-	-	-
FMDV O_1K, 146 S	60	130	270

the peptides still maintained some of their antigenic activity even after denaturation. This may also suggest that the primary structure of the peptides itself contributes to the antigenic activity of the VP_{Thr}CNBr peptides. Several interpretations are possible to explain the weak antigenic activity of the isolated VP_{Thr} peptides. One suggestion may be that there was not a complete set of antigenic determinants in the isolated peptide. The significance of an optimum conformation of the protein for its antigenic activity seems to be of less importance since all experiments have been performed under the same reducing and denaturing conditions. The biologically active fractions were eluted from the Sephadex G-100 column with a partitition factor of 0.44 corresponding to a mol. wt. of 5.200 daltons. Their electrophoretical migration behaviour, however, was

different as shown by (SDS)-polyacrylamide gelelectrophoresis (Fig. 2). It has clearly been demonstrated by Swank and Munkres (16) that the molecular weight of small proteins and peptides and their electrophoretical characteristics were not directly correlated. We therefore consider the reported molecular weight of the VP_{Thr} peptides rather as isolation parameters than as absolute values.

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