A retro-inverso peptide corresponding to the GH loop of foot-and-mouth disease virus elicits high levels of long-lasting protective neutralizing antibodies

JEAN-PAUL BRIAND*, NADIA BENKIRANE*, GILLES GUICHARD*, JOHN F. E. NEWMAN†,
MARC H. V. VAN REGENMORTEL*, FRED BROWN†, AND SYLVIANE MULLER*†

*Institut de Biologie Moléculaire et Cellulaire, Unité Propre de Recherche 9021, Centre National de la Recherche Scientifique, Strasbourg, France; and †Plum Island Animal Disease Center, U.S. Department of Agriculture, Agricultural Research Service, Greenport, NY 11944-0848

ABSTRACT   Peptides corresponding to the immunodominant loop located at residues 135–158 on capsid protein VP1 of foot-and-mouth disease virus (FMDV) generally elicit high levels of anti-peptide and virus-neutralizing antibodies. In some instances, however, the level of neutralizing antibodies is low or even negligible, even though the level of anti-peptide antibodies is high. We have shown previously that the antigenic activity of peptide 141–159 of VP1 of a variant of serotype A can be mimicked by a retro-inverso (all-D retro or retroenantio) peptide analogue. This retro-inverso analogue induced greater and longer-lasting antibody titers than did the corresponding L-peptide. We now show that a single inoculation of the retro-inverso analogue elicits high levels of neutralizing antibodies that persist longer than those induced against the corresponding L-peptide and confer substantial protection in guinea pigs challenged with the cognate virus. In view of the high stability to proteases of retro-inverso peptide analogues and their enhanced immunogenicity, these results have practical relevance in designing potential peptide vaccines.

The first demonstration that small peptides can elicit protective levels of neutralizing antibodies of foot-and-mouth disease virus (FMDV) was obtained by using synthetic peptides corresponding to what is now known as the GH loop of the capsid protein VP1 of FMDV (1, 2). In subsequent work, the fairly low immunogenicity of the peptides was enhanced by using various approaches. It was shown that glutaraldehyde and Cys-Cys polymerization of peptides, liposome presentation, and multiple peptide copies on a polylysine backbone in the multiple antigen presentation (MAP) system are efficient strategies for eliciting high-titer antipeptide responses and antibodies capable of neutralizing the infectious agent (3–5). It was also shown that although the peptide sequence of amino acids 141–160 of the FMDV VP1 fused to the N terminus of β-galactosidase did not produce a more potent immunogen than the synthetic peptide alone (6), immunogenicity of the peptide could be significantly enhanced by expressing it as a fusion protein with hepatitis B core protein to form a virus-like particle (7). Protective immunity induced by peptides linked to keyhole limpet hemocyanin (KLH) as carrier or as a fusion protein in which multiple copies of the peptide were linked to β-galactosidase was demonstrated in guinea pigs, cattle, and pigs (8, 9).

Despite the considerable amount of data gathered on potential synthetic vaccines against foot-and-mouth disease, however, there are still a number of problems associated with the development of a peptide vaccine. A major problem limiting the use of peptides as vaccines, particularly in strategies using the oral and nasal routes, is the instability of natural peptides. Most biologically active peptides are short-lived molecules that are rapidly degraded *in vivo* by proteases. It is possible to overcome this major drawback by replacing standard peptides with pseudopeptides or peptide mimetics that contain changes in the amide bond (—CO—NH—). Such modifications, also referred to as amide bond surrogates, have been widely used in pharmacology, and several peptide analogues endowed with improved biological activity and higher enzymatic stability have been described (10). An important problem encountered with pseudopeptides is the conservation of their biological activity. Although many structure–function studies have been undertaken in the field of peptide drug design, the use of peptidomimetics such as retro-inverso analogues has been described only recently in immunology (11, 12). In retro-inverso peptides, also called all-D retro or retroenantio peptides (13), the direction of the peptide bonds is reversed while the side-chain orientation of the amino acid residues is retained. This is achieved by assembling D-amino acid residues in the reverse order with respect to the original sequence. For example, we have shown that a retro-inverso peptide analogue corresponding to the C-terminal hexapeptide of histone H3 can mimic the antigenic and immunogenic properties of the parent peptide (11, 14). Retro-inverso peptides were also found to serve as valuable probes for detecting antibodies in the serum of autoimmune mice and patients (15). Jameson *et al.* (12) showed that a cyclic retro-inverso analogue of a region of the CD4 receptor of murine T cells was able to inhibit experimental allergic encephalomyelitis and, more recently, it has been demonstrated that a retro-inverso cyclic peptide can act as a competitive inhibitor of IgE binding to their receptor, FcεRI (16). The retro-inverso analogue was also found to inhibit IgE-mediated mast cell degranulation, an *in vitro* model for allergic response. Finally, Guichard *et al.* (17) described that partial retro-inverso peptide analogues of the peptide M 58–66 derived from the influenza virus matrix protein containing a retro-inverso bond between residues 1 and 2 retained the original capacity of binding to HLA-A2 class I molecules. Altogether, these results show the considerable potential of peptidomimetics for developing new generations of therapeutic agents, immunomodulators, and targets useful for immunodiagnostics.

We have recently postulated that because retro-inverso analogues are much more stable than natural peptides to proteolysis (11), they could also be useful alternatives in synthetic vaccines. Retro-inverso peptide analogues of the immunodominant epitope 141–159 of the VP1 protein of two variants of FMDV, serotype A (18), were synthesized and their antigenic and immunogenic properties were tested (19, 20). Compared with the corresponding parent peptides, retro-inverso analogues were shown to induce in rabbits a peptide-specific IgG response of much higher titer that also lasted much longer. Antibodies raised in rabbits against the retro-inverso peptides cross-reacted strongly with virus particles.

In the present work, we have studied the antigenic properties of several retro-inverso peptide analogues of the parent peptide 141–159 of the VP1 of one of these variants. The retro-inverso peptides were tested with guinea pig antisera raised against the parent L-peptide, capsid protein VP1, and FMDV particles, as well as with serum from convalescent guinea pigs infected with the virus. Furthermore, we have tested the ability of retro-inverso peptides to elicit *in vitro* neutralizing antibodies and an *in vivo* protective response in guinea pigs against the cognate virus.

MATERIALS AND METHODS

Virus. The FP variant of FMDV (serotype A, subtype 12) was grown in monolayers of BHK 21 cells (18).

Synthetic Peptides. Seven natural (L-) and retro-inverso peptides corresponding to the VP1 region 141–159 of the virus were used (Table 1). Peptide 141–159 contains both B and T cell epitopes for mice (4) and the highly conserved RGD cell attachment site at residues 145–147 (21). An additional cysteine residue was added at either the N or C termini of the peptides to allow their selective conjugation to BSA or KLH. For coupling, Imject maleimide-activated BSA and KLH from Pierce were used.

Assembly of the parent peptides was carried out on a 25-μmol scale according to classical 9-fluorenylmethoxycarbonyl (Fmoc) methodology on Wang resins for the preparation of peptide acids (—COOH), and Rink amide resins for the preparation of peptide amides (—CONH$_2$). In some cases, the N terminus of the peptide was acetylated (Ac-). After final acid cleavage, each peptide was collected in cold *tert*-butyl methyl ether and centrifuged; the pellets were then washed twice with cold ether. After the last centrifugation, each peptide was dissolved in 10% (vol/vol) acetic acid in water and lyophilized. The 141–159 retro-inverso peptide with blocked N and C termini [NH$_2$-141–159(C)-Ac] was also assembled according to classical Fmoc methodology on a Rink amide resin, using Fmoc-protected D-amino acid derivatives.

In the case of the retro-inverso peptide 141–159 [NH$_2$-(C)141–159-OH], a close mimicry of the parent peptide C terminus was achieved by using a malonate derivative. This peptide was assembled by using *tert*-butoxycarbonyl (Boc) chemistry on a *p*-methylbenzhydralamine resin. Assembly of

the protected peptide chain was carried out on a 100-μmol scale by using the *in situ* neutralization protocol described previously (22). The (*R,S*)-2-isobutylmalonic acid monobenzyl ester (17) was incorporated into the peptide chain as a racemate, thereby generating a pair of diastereoisomers. The peptide was cleaved from the resin by treatment with anhydrous hydrogen fluoride (HF) containing 10% (vol/vol) anisole and 1% (vol/vol) 1,2-ethanedithiol. After removal of HF *in vacuo*, the peptide was extracted from the resin and lyophilized. The crude peptides were then purified on an Aquapore C$_{18}$ ODS column (10 × 100 mm) in a preparative HPLC apparatus (Perkin–Elmer). In the case of the retro-inverso analogue NH$_2$-(C)141–159-OH, the two diastereoisomers were also separated and purified by preparative HPLC. They were identified according to their retention time. Homogeneity of the purified peptides was assessed by analytical HPLC on a 5-μm C$_{18}$ Nucleosil column measuring 4.6 × 150 mm (Macherey–Nagel) and gradient elution [solvent A: 0.1% trifluoroacetic acid (TFA) in water; solvent B: 0.08% TFA in acetonitrile; gradient 5–65% solvent B] in 20 min. Peptide identity was verified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) using a Protein TOF apparatus (Bruker Spectrospin, Bremen, Germany).

Resistance of the parent L-peptide Ac-(C)141–159-OH and the retro-inverso analogue NH$_2$-(C)141–159-OH to trypsin was tested by using the enzyme immobilized on 3.2-mm diameter nylon spheres (11). The specific activity of the spheres was equivalent to 18.5 nmol of *p*-toluenesulfonyl-L-arginine methyl ester hydrolyzed per min per nylon sphere. Protease digestion was initiated by immersing 30 of the spheres in 1 ml of a peptide solution (500 μg of peptide per ml) in Hepes-buffered saline at pH 7.4 and maintained at 25°C under constant agitation. The reaction was stopped at intervals by removing the enzymatic spheres, and the extent of peptide cleavage was estimated by reversed-phase HPLC, using a Beckman instrument with a Nucleosil C$_{18}$ column and a linear gradient of 0.1% TFA and acetonitrile containing 0.08% TFA at a flow rate of 1.2 ml/min. Sensitivity of peptides to trypsin was evaluated from the area of the peak corresponding to the intact peptide remaining at several intervals.

Immunization Schedules and Antisera to Virus Particles, VP1, and Peptides. Female Dunkin–Hartley guinea pigs, approximately 12 weeks old and weighing between 450 and 500 g, were used for immunization. In the initial experiments, the peptides were linked to activated KLH through the N- or C-terminal cysteine residue (see above) and then mixed with aluminum hydroxide gel before 100 μg of peptide was inoculated subcutaneously into guinea pigs. Two animals were immunized once with each peptide conjugate, and serum samples were collected at intervals. In view of the high level of neutralizing antibody elicited, the *in vivo* protective response was tested in guinea pigs that had received one inoculation of only 7.5 μg of the conjugated peptide. These animals were challenged by intradermal inoculation into one hind-foot pad of 10^6 tissue culture infectious doses of the homologous virus.

Table 1.   Amino acid sequences and nomenclature of parent peptides and retro-inverso analogues of the 141–159 peptide of FMDV, FP variant

Name	Sequence
L-Peptide H-141–159(C)-OH	H-G→S→G→V→R→G→D→F→G→S→L→A→P→R→V→A→R→Q→L→C-OH
L-Peptide H-(C)141–159-OH	H-C→G→S→G→V→R→G→D→F→G→S→L→A→P→R→V→A→R→Q→L-OH
L-Peptide H-141–159(C)-NH$_2$	H-G→S→G→V→R→G→D→F→G→S→L→A→P→R→V→A→R→Q→L→C-NH$_2$
L-Peptide Ac-141–159(C)-NH$_2$	CH$_3$CO-G→S→G→V→R→G→D→F→G→S→L→A→P→R→V→A→R→Q→L→C-NH$_2$
RI-Peptide NH$_2$-141–159(C)-Ac	H$_2$N-G←s←G←v←r←G←d←f←G←s←l←a←p←r←v←a←r←q←l←c-COCH$_3$
L-Peptide Ac-(C)141–159-OH	CH$_3$CO-C→G→S→G→V→R→G→D→F→G→S→L→A→P→R→V→A→R→Q→L-OH
RI-Peptide NH$_2$-(C)141–159-OH	H$_2$N-c←G←s←G←v←r←G←d←f←G←s←l←a←p←r←v←a←r←q←(*R,S*)mL-OH

RI, retro-inverso; the arrows (→, ←) indicate the sense of the —CO—NH— bond in the peptide backbone. For conventional reasons, the numbering of residues was maintained in L- and RI-peptides regardless the orientation of the peptide bonds. Lowercase letters indicate D-amino acid residues. m, Malonate.

Antisera to intact particles and VP1 were prepared as described previously (23). Anti-peptide antibodies were raised in guinea pigs against the unconjugated L peptide H-141–159(C)-OH, the same peptide presented as a MAP (5) or conjugated to KLH or BSA through its C-terminal cysteine residue. These were mixed with aluminum hydroxide gel and inoculated subcutaneously.

ELISA. Polyvinyl microtiter plates (Falcon) were coated overnight at 4°C with either 0.1 or 0.2 μM of the various 141–159 peptide analogues dissolved in 0.05 M carbonate buffer, pH 9.6. After three washings of the plates with PBS containing 0.05% Tween (PBS-T), the wells were incubated with PBS-T containing 3% (wt/vol) gelatin for 1 h at 37°C. Antisera were diluted in PBS-T containing 1% gelatin and added to the plates. After 1 h at 37°C and washing with PBS-T, peroxidase-conjugated goat anti-guinea pig IgG (Jackson ImmunoResearch; working dilution 1/5,000 in PBS-T) was added for 30 min at 37°C, followed by incubation with 3,3′,5,5′-tetramethylbenzidine in the presence of H_2O_2. The resulting absorbance was measured at 450 nm. Uncorrected background absorbance of the assays was ≤0.1. Normal guinea pig serum used as control was obtained from Nordic Immunology (Tilburg, The Netherlands).

The capacity of guinea pig antibodies raised against the retro-inverso peptide to recognize virus particles was measured by competitive ELISA as previously described (20). Increasing amounts of virus were first incubated with constant dilutions of antisera for 1 h at 37°C and then overnight at 4°C. The mixtures were subsequently added to peptide-coated wells and the test was performed as for the direct ELISA described above.

***In Vitro* FMDV Neutralization Assay.** Equal volumes of 10-fold virus dilutions and sera diluted 1/50 were incubated at 25°C for 15 min. The mixtures were then added to monolayers of BHK 21 cells grown in wells of microtiter plates. After incubation for 3 days at 37°C in an atmosphere containing 5% CO_2, the cells were stained with crystal violet/formaldehyde. The differences between the titer of the virus alone and the titers of the mixtures give a measure of the neutralizing activity of the sera.

RESULTS

Parent Peptides and Retro-Inverso Analogues. Seven peptides were used in this study (Table 1). They were assembled by using classical solid-phase chemistry. The retro-inverso peptides were synthesized by assembling D-amino acid residues in reverse order with respect to the native sequence. This resulted in a reversed direction of peptide bonds (—NH—CO— instead of —CO—NH— bonds) while approximately maintaining the topology of the side chains when the sequences were read as in the parent peptides (Fig. 1*A*). In such linear peptides, the parent and retro-inverso peptides differ by the reversed position of the charged end groups (Fig. 1*B*). To solve this problem, the simplest approach was to prepare blocked L and retro-inverso peptide analogues [Ac-141–159(C)-NH₂ and NH₂-141–159(C)-Ac], respectively (Table 1). A better approach introduces a *gem*-diaminoalkyl residue at the N terminus and a 2-substituted malonic acid residue at the C terminus of the peptide (Fig. 1*C*) (24). Because the extra N-terminal cysteine residue is not part of the epitope, in this study we simply modified the amino end of this residue by using a carboxamide termination for the retro-inverso peptide NH₂-(C)141–159-OH and an acetyl termination for the corresponding L-peptides [Ac-(C)141–159-OH]. For immunochemical comparisons, the latter acetylated L-peptide is a better control for the retro-inverso analogue. To closely mimic the C terminus of the parent peptide, C2-substituted malonic acid derivatives were chemically synthesized and incorporated during the retro-inverso peptide assembly as racemates, thereby gener-
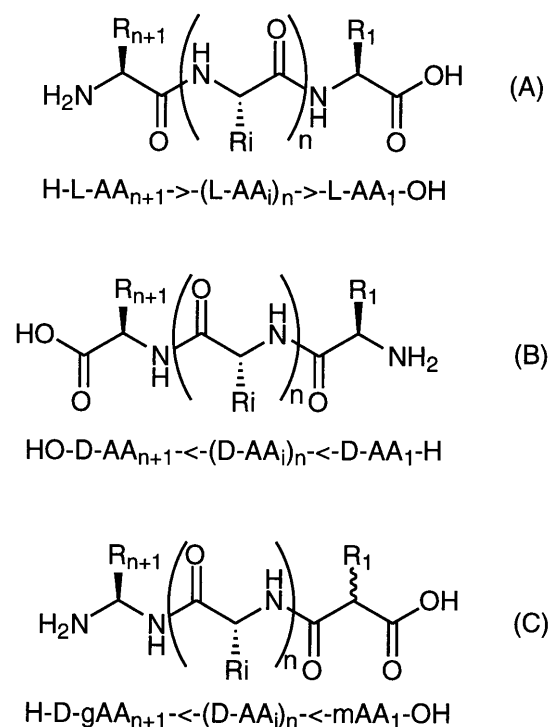

Fig. 1. Schematic representation of a linear L-peptide (*A*), the corresponding end-group unmodified retro-inverso analogue (*B*), and the corresponding end-group-modified retro-inverso analogue (*C*). g, *gem*-diaminoalkane; m, malonate. The arrowheads ($>$, $<$) indicate the sense of the —CO—NH— bond in the peptide backbone.

ating pairs of diastereoisomers (Table 1). The latter were separated and purified by HPLC. The more rapidly eluted isomer is referred to as the RIa peptide and the other as the RIb peptide. Because in all cases the antigenic activity of the RIb analogue was much higher than that of the RIa analogue, only results obtained with the RIb diastereoisomer (referred to as "RI peptide" hereafter) are described. The rate of isomerization at 37°C and pH 7.0 of the retro-inverso analogue NH₂-(C)141–159-OH (RIb) was followed by HPLC. A slow equilibrium was observed after 21 h, leading to about a 60:40 (RIb/RIa) equilibrium mixture of diastereoisomers. The purity of the seven peptides was greater than 90% as checked by analytical HPLC. MALDI-MS analysis gave the expected results for all compounds (data not shown).

Resistance of the Retro-Inverso Peptide NH₂-(C)141–159-OH to Trypsin. Tryptic hydrolysis experiments have been undertaken with the L- and the retro-inverso peptides (Fig. 2). The half-life of the retro-inverso peptide NH₂-(C)141–159-OH was found to be more than 20-fold greater than that of the L-peptide (450 min compared with 20 min).

Antigenic Mimicry of the Region 141–159 of VP1 with Retro-Inverso Peptide Analogues. The capacity of guinea pig antisera raised against intact virus particles, VP1, and L-peptide 141–159 to recognize the retro-inverso peptides 141–159 was tested in ELISA. As shown in Fig. 3*A*, whatever mode of peptide presentation was used to produce anti-peptide antibodies (unconjugated, conjugation to KLH or BSA, MAP; antisera 1–4 in the Fig. 3*A*), the different peptide antisera reacted equally well or better with the retro-inverso analogue NH₂-(C)141–159-OH than with L-peptides H-(C)141–159-OH and Ac-(C)141–159-OH. Similarly, anti-VP1 and anti-virion antisera (antisera 5 and 6 in the figure) reacted strongly with both the retro-inverso and L-peptides 141–159.

The anti-L-peptide H-141–159(C)-OH, anti-VP1, and anti-virus antibodies reacted similarly with the L-peptides blocked at either the N or C terminus (Fig. 3*A*) or with peptides
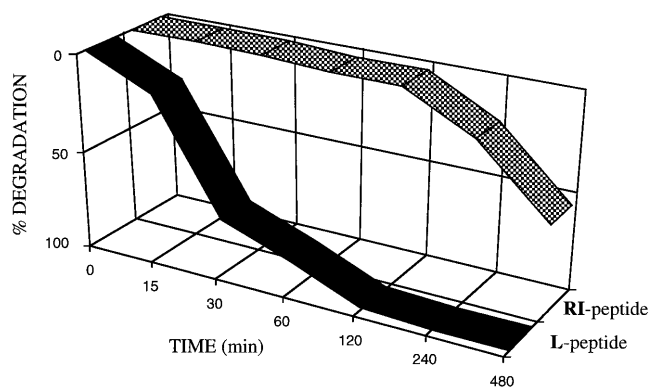
FIG. 2.   Resistance of the parent L-peptide Ac-(C)141–159-OH and the retro-inverso analogue NH_2-(C)141–159-OH to trypsin. Sensitivity of the two peptides was evaluated from the area of the peak corresponding to the intact peptide remaining at several intervals. The half-life of the L-peptide was about 20 min and that of the retro-inverso peptide, about 450 min.

containing a cysteine residue at either the N or C terminus (Fig. 3*B*). In contrast, for all antisera tested (only six are shown in the figure), blocking both the N and C termini of L- and retro-inverso peptides reduced antibody binding considerably (Fig. 3*B*).

By competition ELISA, we found that virus particles in the fluid phase could similarly inhibit the binding between the
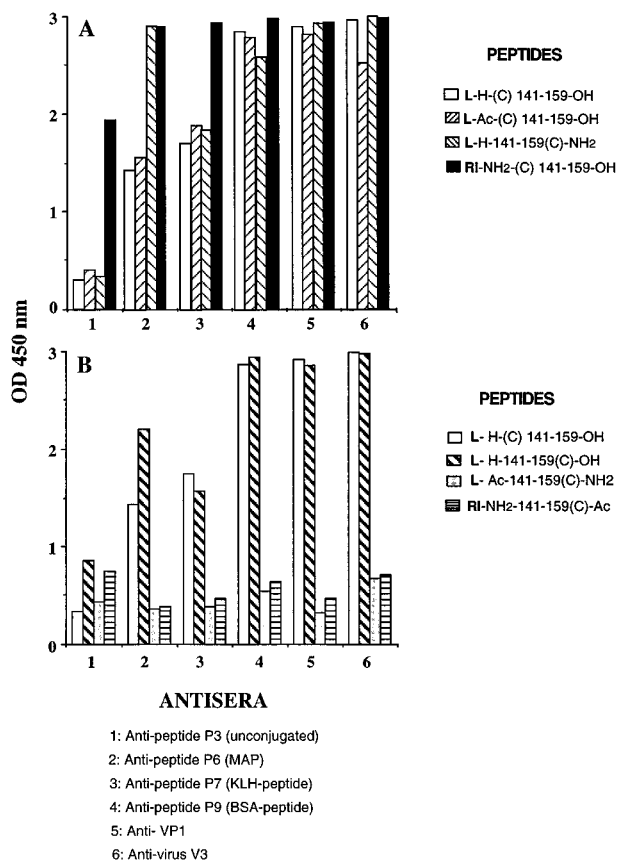

FIG. 3.   Reaction of guinea pig antisera to intact virus particles, VP1, and parent peptide 141–159(C) with parent and retro-inverso peptides. Serum samples were tested in ELISA using 0.1 μM nonconjugated peptide for coating plates. Anti-peptide sera were raised against L-peptide H-141–159(C)-OH, unconjugated (P3), presented as a MAP (P6), or conjugated to KLH (P7) or BSA (P9) through its C-terminal cysteine. The antisera were diluted 1/20,000. Reaction with normal guinea pig serum was insignificant (OD values <0.10).

retro-inverso NH_2-(C)141–159-OH peptide analogue and antibodies to L-peptide H-(C)141–159-OH and to retro-inverso NH_2-(C)141–159-OH peptide (Fig. 4). This result indicates that the retro-inverso peptide can efficiently mimic the cognate sequence in the virus.

Immunogenicity of Retro-Inverso Peptide Analogue and Induction of Neutralizing Antibodies. We have shown previously (19) that the retro-inverso peptide NH_2-(C)141–159-OH covalently coupled to small unilamellar liposomes containing monophosphoryl lipid A as adjuvant elicited in rabbits a long-lasting antibody response with antibody titers higher than those obtained in animals inoculated with the corresponding L-peptide. These antibodies cross-reacted strongly with L-peptides and with virus particles (20). To determine whether antibodies to the retro-inverso peptides can neutralize the virus, we immunized guinea pigs with the various analogues covalently conjugated to KLH and followed the *in vitro* neutralization titers of the sera taken at various intervals for 362 days. The animals received a single dose of 100 μg of peptide with aluminum hydroxide gel as adjuvant. As shown in Fig. 5, the level of the response to the retro-inverso peptide NH_2-(C)141–159-OH was similar to that obtained with the L-peptides H-141–159(C)-NH_2 and H-(C)141–159-OH up to around 50 days after the inoculation. However, compared with the response to the L-peptides, the response against retro-inverso NH_2-(C)141–159-OH peptide continued to increase beyond 50 days (the neutralizing indices are at least 10-fold higher at 100 days). In the samples collected 362 days after inoculation of the animals, the neutralizing indices of the sera from guinea pigs that received the retro-inverso peptide were still significantly higher than those of the sera from responder animals inoculated with the L-peptides.

The sequential bleedings were tested in ELISA to measure the level of antibodies obtained after a single injection of 100 μg of L- and retro-inverso peptides and to determine whether there was a correlation between the ELISA antibody levels and titers measured in the neutralization assays. All the animals
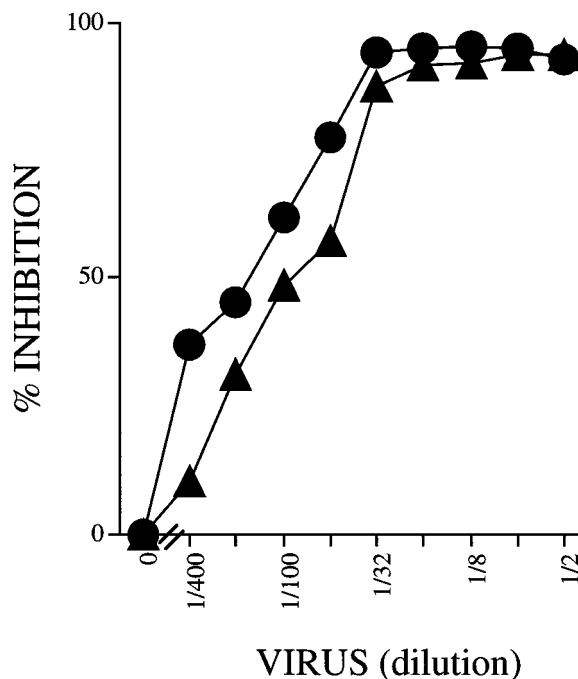

FIG. 4.   Inhibition of the ELISA reaction between guinea pig antibodies to L- or retro-inverso peptide 141–159 and the retro-inverso peptide by increasing concentrations of virus. ▲, Anti-L-peptide H-(C)141–159-OH; ●, anti-retro-inverso peptide NH_2-(C)141–159-OH. Unconjugated retro-inverso peptide NH_2-(C)141–159-OH (0.2 μM) was used for coating plates.
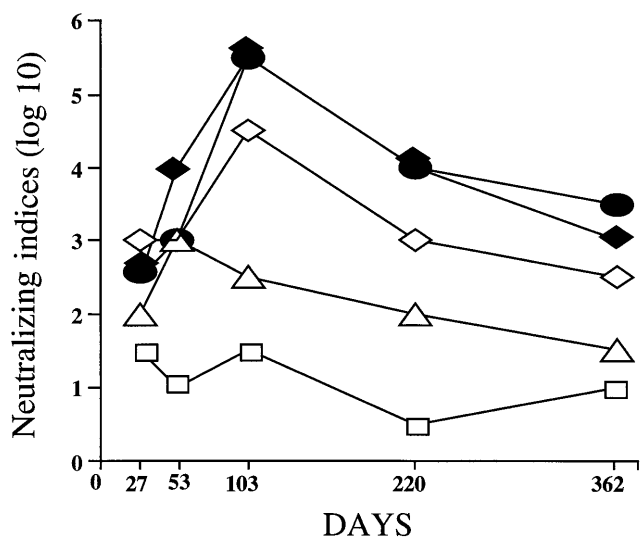
FIG. 5. *In vitro* neutralizing activity in sequential bleedings collected from guinea pigs that received one inoculation of 100 µg of L- and retro-inverso peptides 141–159. Peptides used for inoculation: △, L-peptide H-(C)141–159-OH; ◇ and □, L-peptide H-141–159(C)-NH$_2$ (two animals); ● and ◆, retro-inverso peptide NH$_2$-(C)141–159-OH (two animals). A neutralizing index of 2.0 logs means that a 1/100 dilution of the antiserum reduces the titer of the homologous virus by 2 log$_{10}$ units—e.g., from 10^6 to 10^4.

immunized with the L- and retro-inverso peptides responded; the maximum antibody response measured in ELISA was usually observed around day 30 and the maximum homologous titers were around 11,000-16,000 in the case of guinea pigs immunized against the L-peptide H-(C)141–159-OH and retro-inverso NH$_2$-(C)141–159-OH peptide. ELISA antibody titers dropped sharply 7–8 weeks after inoculation of both L- and retro-inverso peptides. In these animals, we found no correlation between ELISA antibody titers and neutralization indices. The kinetics of appearance of neutralizing antibodies (whose titers increased with time up to around 100 days) and anti-peptide antibodies measured in ELISA (whose titers decreased rapidly with time) were not associated (data not shown).

We also tested the immunogenicity of the KLH-conjugated L- and retro-inverso peptides that were blocked at both N- and C-terminal extremities. These peptides induced a good antibody response measurable in ELISA with the homologous peptides and with the L-peptide H-141–159(C)-NH$_2$, but the antibodies reacted very weakly with the unblocked peptide H-141–159(C)-OH. Neither gave a neutralizing antibody response. This is in accordance with the ELISA results described above (Fig. 3*B*), showing that peptides blocked at both ends react poorly with anti-virion, anti-VP1, and anti-peptide sera containing high levels of antibodies reacting with unblocked peptides or peptides blocked at only one end.

***In Vivo* Protection assays.** To determine whether the neutralizing antibodies elicited by the retro-inverso peptide NH$_2$-(C)141–159-OH were protective, nine guinea pigs were inoculated with 7.5 µg of peptide linked to activated KLH. At 81, 102, or 136 days after inoculation, separate groups of three animals were challenged by intradermal inoculation into one hind-foot pad of 10^6 tissue culture infectious doses of the homologous virus. The sera had a range of neutralizing indices of 2.0 to ≥3.0 log$_{10}$ units at a 1/100 dilution. Whereas control animals that received no peptide developed lesions on the inoculated foot and other feet, immunized animals with neutralizing indices of 3.0 log units or greater (three animals) did not develop lesions, even at the site of inoculation. Those with neutralizing indices of 2.0–2.5 log units (six animals) developed lesions at the site of inoculation but no lesions appeared

on the other feet. In comparative assays with three guinea pigs inoculated with 7.5 µg of L-peptide Ac-(C)141–159-OH, the animals developed serum neutralizing indices of 1.5–2.0 log units at day 136 and were not protected against challenge infection.

DISCUSSION

The results described in this study emphasize the cross-reactive properties of natural L-peptides and the corresponding retro-inverso (all-D retro) isomers. We were able to confirm in the guinea pig model our earlier experimental data obtained in rabbits, which showed that the end-group modified retro-inverso isomer 141–159 mimicked the parent peptide (19, 20). Moreover, we show here that, compared with the L-peptide, the retro-inverso peptide NH$_2$-(C)141–159-OH elicited much higher levels of neutralizing antibodies and that these were maintained for a longer period than those elicited by the corresponding L-peptide. This may be accounted for by the much greater resistance of the retro-inverso peptide to proteolytic enzymes *in vivo*, because we found that the retro-inverso peptide analogue is much more resistant to hydrolysis by trypsin than the corresponding L-peptide. Furthermore, we found that a single inoculation of only 7.5 µg of the retro-inverso analogue NH$_2$-(C)141–159-OH induces a protective response against a severe challenge infection. The level of protection was directly correlated with the level of neutralizing antibodies in the immunized animals. These results show that a highly stable retro-inverso analogue may provide the basis for the development of an efficient peptide-based vaccine.

Although the mechanisms involved in the cell-mediated immune response to such analogues still remain to be elucidated, the present findings allow us to better understand the structural requirements of retro-inverso peptide analogues for inducing cross-reactive antibodies and a neutralizing and protective immune response against FMDV. It has been shown recently that the structure in trifluoroethanol (TFE) of the L-and retro-inverso peptides 141–159 (FMDV serotype A12, SL variant) differ considerably (25). However, as discussed previously (26), it seems likely that the "active" retro-inverso peptide described in this study is topochemically related and antigenically equivalent to the parent peptide because the latter is present as random coils in aqueous solution and adopts a partial α-helical conformation only in TFE (25, 27).

Antibody cross-recognition of retro-inverso analogues of a parent peptide has been described for both short and longer (up to 21 residues) linear sequences (11, 15, 19, 28). In the case of the retro-inverso analogues of one peptide from a ribonucleoprotein antigen (15) and of a 15-mer peptide able to bind interleukin 2 (28), it was found that the retro-inverso analogues with unblocked reversed termini were equally well or better recognized than the respective L-peptides. This suggests that for these particular peptides, reversal of the end groups did not affect their antigenicity. However, as it is well documented that many retro-inverso analogues of biologically active peptides with reversed termini are totally inactive, appropriate end group modifications have been introduced to solve this problem (11, 19, 29). In the present work, C2-substituted malonic acid residues have been introduced at the C terminus of the retro-inverso analogues to closely mimic the C terminus of the corresponding parent peptides, while the N terminus of analogues has been blocked by amidation. As control, in the respective L-peptides the N terminus has been blocked by acetylation. Another strategy adopted for certain peptides in previous work (15) was to block both N and C termini. In some cases, this approach has been found to be highly effective, but in other cases blocking both extremities of L-peptides was detrimental to their antigenic activity. In the present study, we have found that the parent peptide 141–159 with both the N and C termini blocked by acetylation and carboxamidation,

respectively, was at best only poorly recognized by anti-peptide, anti-protein, and anti-virus antibodies, and it did not induce neutralizing antibodies.

Our experiments show that anti-virion, anti-VP1, and anti-L-peptide guinea pig antibodies cross-react strongly with the retro-inverso peptide analogue of an immunodominant epitope of FMDV and that a very low dose of the latter analogue induces a long-lasting and fully protective neutralizing response against viral infection. The generality of this antigenic mimicry, even though it does not seem to apply to the "handedness" of the helices in helical peptides (26), could be extended to peptides in loop or cyclic conformation. The stability and cross-antigenicity of viral retro-inverso peptides provide a novel and interesting route to the development of a new generation of synthetic vaccines.

Note Added in Proof. Evidence for the topological similarity between an L- and a retro-inverso cyclic peptide has been presented recently (30).

1. Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G., Lerner, R. A., Rowlands, D. J. & Brown, F. (1982) *Nature (London)* **298,** 30–33.
2. Pfaff, E., Mussgay, M., Böhm, H. O., Schulz, G. E. & Schaller, H. (1982) *EMBO J.* **1,** 869–874.
3. Bittle, J. L., Worrell, P., Houghten, R. A., Lerner, R. A., Rowlands, D. J. & Brown, F. (1984) in *Modern Approaches to Vaccines*, eds. Chanock, R. M. & Lerner, R. A. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 103–107.
4. Francis, M. J., Fry, C. M., Rowlands, D. J., Brown, F., Bittle, J. L., Houghten, R. A. & Lerner, R. A. (1985) *J. Gen. Virol.* **66,** 2347–2354.
5. Francis, M. J., Hastings, G. Z., Brown, F., McDermed, J., Lu, Y.-A. & Tam, J. P. (1991) *Immunology* **73,** 249–254.
6. Winther, M. D., Allen, G., Bomford, R. H. & Brown, F. (1986) *J. Immunol.* **136,** 1835–1840.
7. Clarke, B. E., Newton, S. E., Carroll, A. R., Francis, M. J., Appleyard, G., Syred, A. D., Highfield, P. E., Rowlands, D. J. & Brown, F. (1987) *Nature (London)* **330,** 381–384.
8. Di Marchi, R., Brooke, G., Gale, C., Cracknell, V., Doel, T. & Mowat, N. (1986) *Science* **232,** 639–641.
9. Broekhuijsen, M. P., Van Rijn, J. M. M., Blom, A. J. M., Pouwels, P. H., Enger-Valk, B. E., Brown, F. & Francis, M. J. (1987) *J. Gen. Virol.* **330,** 3137–3143.
10. Fauchère, J.-L. & Thurieau, C. (1992) *Adv. Drug Res.* **23,** 127–159.
11. Guichard, G., Benkirane, N., Zeder-Lutz, G., Van Regenmortel, M. H. V., Briand, J. P. & Muller, S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9765–9769.
12. Jameson, B. A., McDonnell, J. M., Marini, J. C. & Korngold, R. (1994) *Nature (London)* **368,** 744–746.
13. Chorev, M. & Goodman, M. (1995) *Trends Biotechnol.* **13,** 438–445.
14. Benkirane, N., Guichard, G., Van Regenmortel, M. H. V., Briand, J.-P. & Muller, S. (1995) *J. Biol. Chem.* **270,** 11921–11926.
15. Briand, J. P., Guichard, G., Dumortier, H. & Muller, S. (1995) *J. Biol. Chem.* **270,** 20686–20691.
16. McDonnell, J. M., Beavil, A. J., Mackay, G. A., Jameson, B. A., Korngold, R., Gould, H. J. & Sutton, B. J. (1996) *Nat. Struct. Biol.* **3,** 419–425.
17. Guichard, G., Connan, F., Graff, R., Ostankovitch, M., Muller, S., Guillet, J. G., Choppin, J. & Briand, J. P. (1996) *J. Med. Chem.* **39,** 2030–2039.
18. Rowlands, D. J., Clarke, B. E., Carroll, A. R., Brown, F., Nicholson, B. H., Bittle, J. L., Houghten, R. A. & Lerner, R. A. (1983) *Nature (London)* **306,** 694–697.
19. Muller, S., Guichard, G., Benkirane, N., Brown, F., Van Regenmortel, M. H. V. & Briand, J. P. (1995) *Peptide Res.* **8,** 138–144.
20. Benkirane, N., Guichard, G., Briand, J.-P., Muller, S., Brown, F. & Van Regenmortel, M. H. V. (1996) in *Developments in Biological Standardization*, ed. Brown, F. (Karger, Basel), Vol. 87, pp. 283–291.
21. Fox, G., Parry, N. R., Barnett, P. V., McGinn, B., Rowlands, D. J. & Brown, F. (1989) *J. Gen. Virol.* **70,** 625–637.
22. Neimark J. & Briand J.-P. (1993) *Peptide Res.* **6,** 219–228.
23. Meloen, R. H., Rowlands, D. J. & Brown, F. (1979) *J. Gen. Virol.* **45,** 761–763.
24. Goodman, M. & Chorev, M. (1979) *Acc. Chem. Res.* **12,** 1–7.
25. Carver, J. A., Esposito, G., Viglino, P., Fogolari, F., Guichard, G., Briand, J.-P., Van Regenmortel, M. H. V., Brown, F. & Mascagni, P. (1997) *Biopolymers* **41,** 569–590.
26. Guichard, G., Muller, S., Van Regenmortel, M. H. V., Briand J.-P., Mascagni, P. & Giralt, E. (1996) *Trends Biotechnol.* **14,** 44–45.
27. Siligardi, G., Drake, A. F., Mascagni, P., Rowlands, D., Brown, F. & Gibbons, W. A. (1991) *Eur. J. Biochem.* **199,** 545–551.
28. Verdoliva, A., Ruvo, M., Cassani, G. & Fassina, G. (1995) *J. Biol. Chem.* **270,** 30422–30427.
29. Chorev, M. & Goodman, M. (1993) *Acc. Chem. Res.* **26,** 266–273.
30. McDonnell, J. M., Fushman, D., Cahill, S. M., Sutton, B. J. & Cowburn, D. (1997) *J. Am. Chem. Soc.* **119,** 5321–5328.