Exploration of Requirements for Peptidomimetic Immune Recognition

ANTIGENIC AND IMMUNOGENIC PROPERTIES OF REDUCED PEPTIDE BOND PSEUDOPEPTIDE ANALOGUES OF A HISTONE HEXAPEPTIDE*

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We present a detailed analysis of the antigenic and immunogenic properties of a series of very stable peptidomimetics of a model hexapeptide corresponding to the C-terminal residues 130–135 of histone H3. Five pseudopeptide analogues of the natural sequence IRCERA were synthesized by systematically replacing, in each analogue, one peptide bond at a time by a reduced peptide bond ψ(CH2–NH). Three important features of the resulting analogues were examined. First, the analogues were tested in a biosensor system for their ability to bind monoclonal antibodies generated against the parent natural peptide, and their kinetic rate constants were measured. The results show that reduced peptide bond analogues can very efficiently mimic the parent peptide. The position of reduced bonds which were deleterious for the binding was found to depend on the antibody tested, and one monoclonal antibody recognized all five analogues. The equilibrium affinity constant toward reduced peptide bond analogues of four antibodies of IgG1 isotype induced against the parent hexapeptide was higher (up to 670 times) than toward the homologous peptide. Second, immunogenic properties of the five analogues were studied, and it was found that polyclonal antibodies induced against analogues in which ψ(CH2–NH) bonds were introduced between residues 130–131, 131–132, and 132–133 (R1-R2, R2-R3, and R3-R4) cross-reacted strongly with the cognate protein H3. Third, we tested the protease resistance of analogues. Altogether, the results provide a strong support for the potential applicability of reduced peptide bond pseudopeptides as components of synthetic vaccines and open a new field for the development of immunomodulatory agents.

Potential applications of peptidomimetics cover many aspects of basic immunology including synthetic vaccines, immunodiagnostics, and the development of new generations of immunomodulators. The possibility of controlling B and T cell recognition has been recently explored by using peptidomimetics, i.e. peptide analogues in which the peptide bond, but not the residue itself, has been changed. We have shown that a retro-inverso peptide (also called all-o-retro peptide) analogue, corresponding to the C-terminal hexapeptide of histone H3, can mimic the antigenic and immunogenic properties of the parent peptide (1, 2). Antibodies of most isotypes raised against the d-enantiomer of this peptide recognized the retro (all-l-retro) analogue but not the parent peptide nor the retro-inverso peptide. Retro-inverso peptides were also found to serve as targets for antibodies produced by lupus mice and by patients with rheumatic autoimmune diseases (3). More recently, retro-inverso peptide analogues of the immunodominant epitope 141–159 of the VP1 protein of two variants of the foot-and-mouth disease virus (FMDV)1 were shown to induce longer lasting and higher antibody titers in immunized animals than did the corresponding l-peptide (4). This peptide encompasses both a B- and T-epitope. Finally, Jameson et al. (5) 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. This illustrates that pseudopeptides can have considerable immunotherapeutic effects.

Sequence identification of a large number of peptides naturally associated with MHC molecules, as well as peptide-binding investigations, have allowed determination of allele-specific peptide motifs for several dozen MHC molecules. Extensive studies have involved amino acid substitutions, i.e. modifications which alter only the side chain, to better approach both the requirement for anchor residues and the importance of non-anchor residues that can make contact with the MHC groove. All of this information (e.g. see Ref. 6 for a review) helped in the design of antigen analogues containing subtle changes whose properties are to be recognized by both antigen-presenting cells and T cell receptors and to induce signal transduction in such a way that either T cell activation or, on the contrary, T cell down-regulation will take place (7–9). Pharmacologically, further studies including backbone modifications of the natural sequence are required to optimize potency of such ligands of the immune cell receptors leading to peptidomimetics with increased stability. In this context of immunological drug design, we have recently examined the ability of reduced peptide bond pseudopeptide analogues (which contain a CH2–NH bond instead of the CO–NH peptide bond) to bind a murine class I molecule, K0. Eight pseudopeptide analogues of the antigenic nonapeptide 252–260 derived from Plasmodium berghei were synthesized by systematically replacing one peptide bond at a time by a reduced peptide bond. We have found that the five analogues containing a reduced bond between P1-P2, P2-P3, P4-P5, P5-P6, and P6-P7, could efficiently mimic the parent peptide in binding to a recombinant single chain SC-K0

1 The abbreviations used are: FMDV, foot-and-mouth disease virus; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate; MPLA, mono-phosphoryl lipid A; mAb, monoclonal antibody; MHC, major histocompatibility complex; HPLC, high performance liquid chromatography.

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molecule, while the introduction of the reduced bond between P$_2$-P$_4$, P$_4$-P$_6$, and P$_8$-P$_9$ was deleterious for SC-Kb binding (10). These results illustrate the potential usefulness of such peptide analogues for the development of stable immunomodulators and peptide-based cytotoxic T lymphocyte vaccines.

To further explore the possibility of using reduced-peptide bond peptidomimetics in vaccinology, it was important to study both antigenic and immunogenic properties of such analogues. In particular, it was necessary to demonstrate that reduced peptide bond peptide analogues can elicit specific antibodies and that generated antibodies can cross-react with the cognate protein. In the present work, we have analyzed the antigenic and immunogenic properties of a series of analogues of model peptide of sequence IRGERA corresponding to the COOH-terminal residues 130–135 of histone H3. Pseudopeptide analogues, whose chemical synthesis has been described elsewhere (11), were obtained by systematically replacing, in each analogue, one peptide bond at a time by a reduced peptide bond.

Three important features were analyzed in this study. First, the different analogues were tested in a biosensor system for their ability to bind monoclonal antibodies generated against the parent peptide, and both their kinetic rate and equilibrium affinity constants were measured. Second, their immunogenicity was investigated. Finally, their resistance to trypsin was evaluated using calibrated enzymatic nylon spheres. Their antigenic and immunogenic properties, associated with their remarkable high resistance to proteolytic digestion, are fundamental characteristics that should favor the use of reduced peptide bond pseudopeptides for vaccination, immunomodulation, and peptide drug delivery.

MATERIALS AND METHODS

Parent Peptide and Pseudopeptide Analogues—Table I shows the sequences of five analogues of parent peptide IRGERA with the abbreviated names used in the text. The synthesis, purification, and analysis of these analogues have been described in detail elsewhere (11). Briefly, the reduced peptide bond was formed by reductive amination of N-butoxycarbonyl-protected α-amino aldehydes in the presence of NaBH$_4$. Fragment condensation and stepwise elongation of the peptide chains using benzotriazolyl N-oxysuccinimidyldimethylamino phosphonium hexafluorophosphate as a coupling agent led to the fully protected nonpeptide. Peptide cleavage and N-terminal C purification of the C$_{18}$ column, the final products were identified by 500 MHz 1H NMR spectroscopy and fast atom bombardment mass spectroscopy. Their purity was ≥95%. In addition, two truncated peptides were synthesized. They correspond to sequences IRG and GERA. All peptides contain a CCG arm at their N-terminal end to allow selective conjugation of the peptides to bovine serum albumin (BSA) and to enhance accessibility of peptides bound to a carrier. Peptides were coupled to BSA by means of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) as described previously (12), and the yield of coupling was obtained by spectrophotometrically the release of 2-thiopyridone from SPDP-derivated BSA on interaction with cysteine-containing peptides (12).

Anti sera and Monoclonal Antibodies—Rabbit antisera TRI directed against whole histone H3 and rabbit antisera MEL and GIZ directed against whole histone H3 and rabbit antisera MEL and GIZ reacted with ammonium sulfate at 50% saturation. After extensive dialysis, the complexes were dissolved and made up to their original volume in phosphate buffer, pH 7.4. Their purity was checked by 12% SDS-polyacrylamide electrophoresis. When required, antibodies were further purified by protein A-Sepharose chromatography. The antibody concentration was determined by optical density measurement at 280 nm using an absorption coefficient of 1.4. The protein concentration was also determined using the Bio-Rad protein assay. Mouse antisera to the parent peptide and reduced peptide bond pseudopeptide analogues were obtained from BALB/c mice immunized with small unilamellar liposome-associated peptides following the procedure described previously (13, 14). Liposomes contained monophosphoryl lipid A (MLP) as an adjuvant. The injections were given at intervals of 3 weeks. Blood was withdrawn from the retroorbital venus plexus 5 days after each injection. Bleedings were then repeated at 30-day intervals over a period of several months after the last injection. As a control, a preliminary bleeding of each mouse was performed before the first injection.

ELISA and Kinetic Analysis of mAb Binding—The direct ELISA procedure and inhibition of direct ELISA experiments used to measure the binding of antibodies from rabbit and mouse sera was as described previously (13). Relative affinity of mAbs with peptide analogues was determined using biosensor technology. The procedure used for equilibrium affinity measurements in the BIAcore™ (Pharmacia Biosensor AB, Uppsala, Sweden) has been described previously (2). This system allows the measurement of molecular interaction in real time and without labeling of the reactants. Both the general procedure used to measure the antibody kinetic constants and the theory of kinetic measurements using the BIAcore biosensor system have been recently described in a review article from this laboratory (15).

Resistance to Trypsin—Resistance of the parent peptide and five peptide analogues to trypsin was tested as described previously (1) using the proteolytic enzyme immobilized on 3.2-mm diameter nylon spheres (16). The specific activity of the enzymatic spheres was equivalent to 18.5 mmol of p-toluensulfonfyl-l-arginine methyl ester hydrolyzed per min per nylon sphere. Protease digestion was initiated by immersing 15 enzymatic spheres into 1 ml of a peptide solution (150 μg/ml) maintained at 25 °C under constant agitation. The digestion was performed in hepes-buffered saline at pH 7.4 for 5–240 min. The reaction was stopped by removing the enzymatic spheres. Peptide cleavage was evaluated immunochromatically in the BIAcore by measuring the capacity of the remaining peptide to compete with the binding of mAb 4x11 (4 μg/ml) to the parent peptide immobilized on the dextran matrix. The maximum inhibitory capacity (100% inhibition) was established individually for each peptide analogue in the absence of trypsin. Alternatively, peptide cleavage was monitored by HPLC. Analytical HPLCs were run on a Beckman instrument (Gagny, France) with a Nucleosil C18 column 5 μm (3.9 × 150 mm) using a linear gradient of (A) 0.1% trifluoroacetic acid and (B) acetonitrile containing 0.08% trifluoroacetic acid, at a flow rate of 1.2 ml/min. The peptide solutions were analyzed by reversed phase HPLC (linear gradient, 1–21% B, 20 min). Sensitivity of peptides to trypsin was evaluated by calculating the area of the peak corresponding to the intact peptide remaining after incubating each analogue with enzymatic beads.

RESULTS

Recognition of Peptide Analogues by Rabbit and Mouse Anti-peptide and Anti-parent Protein Antibodies—We have shown previously that, using BSA-conjugated peptides as antigen for coating ELISA plates, antibodies from several antisera raised against the parent peptide IRGERA and histone H3 reacted almost equally well with the parent peptide and with peptide analogues RVR, RVG, and GFE containing reduced peptide bonds between residues 130–131 (R1-R2), 131–132 (R2-R3), and 132–133 (R3-R4), respectively (11). On the other hand, anti-peptide and anti-H3 antibodies reacted very slightly or not at all with analogous EVR and RVA containing reduced peptide bonds between residues 133–134 (R4-R5) and 134–135 (R5-R6), respectively. The same results were obtained with mouse antibodies raised against the parent peptide coupled to small unilamellar liposomes containing MPLA (not shown). To further analyze the antigenic properties of the reduced peptide bond analogues, we performed in the present study a competitive ELISA test in which peptide analogues were used as free inhibitors. In Table II, we show that, as found in the direct
ELISA format, the parent peptide and analogues 1FV, RVG, and GVE are the only peptides able to significantly inhibit the ELISA reaction between the parent peptide and antibodies raised against the parent peptide and the cognate protein H3, while the analogues EYR and RVA failed to inhibit or inhibited poorly the antibody binding. From these data, we could thus confirm our preliminary observations showing that the replacement by a reduced bond of the natural peptide bond between residues R4-R5 and R5-R6 in analogues EYR and RVA significantly affects their recognition by rabbit and mouse polyclonal antibodies, while antibody binding was not or only slightly affected by the introduction of a reduced bond between residues R1-R2, R2-R3, and R3-R4.

The fact that the replacement of a peptide bond by a reduced peptide bond in analogues 1FV, RVG, and GVE has apparently very little effect on the reactivity of both rabbit and mouse polyclonal antibodies in direct and inhibition ELISA format may, however, appear somewhat dubious. We may indeed argue that this lack of effect is apparent only because in fact antisera studied contain very few antibodies reacting with the N-terminal domain IRG of the sequence IRGERA. We thus tested in inhibition assays the ability of the antisera MEL, GIZ, and TRI to react with two shorter peptides, namely peptides IRG and ERA, and found that in their free form both peptides could inhibit the recognition of polyclonal antibodies to the parent peptide IRGERA (Table II). The argument is thus not valid since antibodies reacting with the N-terminal part of the IRGERA peptide are present in the antisera. Moreover, because a peptide similar to the parent peptide except that it may, however, appear somewhat dubious. We may indeed argue that this lack of effect is apparent only because in fact antisera studied contain very few antibodies reacting with the N-terminal domain IRG of the sequence IRGERA. We thus tested in inhibition assays the ability of the antisera MEL, GIZ, and TRI to react with two shorter peptides, namely peptides IRG and ERA, and found that in their free form both peptides could inhibit the recognition of polyclonal antibodies to the parent peptide IRGERA (Table II). The argument is thus not valid since antibodies reacting with the N-terminal part of the IRGERA peptide are present in the antisera. Moreover, because a peptide similar to the parent peptide except that it contained no CGG arm showed the same ability to inhibit the binding (Table II), the observed inhibition with truncated peptides could not be ascribable to the CGG arm itself. This fact could also be deduced by the lack of inhibition found with analogues EYR and RVA, which both contain a CGG arm.

**Table II**

| Peptides used as inhibitors (10 μM) | Inhibition of the binding between IRGERA and antibodies induced against: |
|-----------------------------------|---------------------------------------------------------------------|
|                                   | Parent peptide  | IRGERA | Protein H3 |
|                                   | MEL    | GIZ    | TRI     |
| Parent peptide                    | 80.6   | 73.6   | 89.5    |
| (CGG)IRGERA                      |         |        |         |
| Analogues                         | 77.8   | 76.6   | 89.1    |
| (CGG)IRGERA                      |         |        |         |
| (CGG)1FV                         | 88.9   | 75.3   | 91.7    |
| (CGG)1FV                         |         |        |         |
| (CGG)RVG                         | 92.5   | 42.0   | 85.9    |
| (CGG)RVG                         |         |        |         |
| (CGG)GVE                         | 0      | 36.3   | 0       |
| (CGG)GVE                         |         |        |         |
| Truncated peptides                | 93.3   | 76.2   | 82.0    |
| (CGG)IRG                         |         |        |         |
| (CGG)ERA                         | 77.5   | 75.0   | 76.2    |
| Parent peptide without CGG arm   | 78.8   | 83.8   | 88.3    |
| CGG arm                          |         |        |         |
| IRGERA                           |         |        |         |

Recognition of Peptide Analogues by mAbs to the Parent Peptide IRGERA—Four mAbs called 4x8, 4x10, 4x11, and 4x12 induced against the parent peptide (CGG)IRGERA were tested with peptide analogues. All mAbs but 4x12 have been described previously (2). All four mAbs belong to the IgG1 subclass. Two additional mAbs were tested, namely mAb 13x18 (IgG3a) generated against a retro-inverso (all-D) analogue and mAb 12x10 (IgG1) generated against a retro (all-L) analogue (2). We determined the kinetic rate and equilibrium affinity constants of these six mAbs for the five immobilized peptide analogues and the parent peptide. As shown in Table III, both mAbs 4x8 and 4x10 reacted with the parent peptide and peptide analogues 1FV, RVG, and GVE. Very interestingly, mAb 4x8 had a higher equilibrium affinity value \( K_a \) for the GVE analogue and mAb 4x10 had a higher \( K_a \) for the 1FV analogue compared with their respective \( K_a \) values for the parent peptide. In the case of mAb 4x8, this was mainly due to a higher association constant \( (k_a) \); while in the case of mAb 4x10, this was due to both a slightly higher \( k_a \) and a slightly lower dissociation constant \( (k_d) \).

mAb 4x11 recognized all five analogues. Still much more striking than the heterospecificity observed with mAbs 4x8 and 4x10, mAb 4x11 showed a much higher affinity for analogues 1FV, RVG, and GVE than for the parent peptide (\( K_a \) values increased 673, 353, and 473 times, respectively). mAb 4x11 also cross-reacted with analogues EYR and RVA with \( K_a \) values increased 83 and 63 times, respectively, compared with the \( K_a \) value for the parent peptide. Dissociation rate constants of mAb 4x11 with respect to all analogues were relatively similar (38–129 \( 10^3 \) s\(^{-1} \)). On the other hand, the increased affinities for reduced peptide bond analogues were mainly due to higher \( K_a \) values (increased 40–867 times).

Finally, mAb 4x12 also showed an interesting reactivity pattern. It did not react with peptide analogues 1FV, RVG, and GVE, whereas it bound both peptide analogues EYR and RVA. Again, mAb 4x12 was found to better react with the latter analogues than with the parent peptide. The \( K_a \) values of mAb 4x12 for analogues EYR and RVA were increased 4 and 32 times with respect to the \( K_a \) value for the parent peptide. The association rate constant of mAb 4x12 for analogue RVA was increased 10 times.

mAb 13x18 (generated against the retro-inverso peptide) was introduced in this study because we found previously that it reacted equally well with the retro-inverso analogue and the parent IRGERA peptide (2). In the present study, mAb 13x18 was found to react, although less well than with the parent peptide, with all five reduced peptide bond analogues. mAb 13x18 was found more sensitive to changes occurring in the last C-terminal residues of the peptide (analogues EYR and RVA).

mAb 12x10 (anti-retro peptide) was introduced as a negative control in the present study. As expected, it did not react with any of the reduced peptide bond analogues. This mAb was shown previously (2) to be unable to bind the parent peptide IRGERA; it recognized only the homologous retro peptide and the all-L analogue.

**Ability of Mice to Produce Antibodies against Reduced Peptide Bond Analogues and Cross-reaction of These Antibodies with the Parent Peptide and Histone H3—**Groups of four BALB/c mice were injected with the various peptide analogues as well as with the parent peptide after conjugation to liposomes containing MPLA. All five peptide analogues induced an antibody response in immunized mice. An IgG response could be generally demonstrated by ELISA from bleeding three onward and, depending on the peptide used as immunogen, antibody activity decreased from bleeding six to seven (i.e. 107 and 133 days after the last injection) (Fig. 1). In all mice, the pattern of antibody production was very similar in terms of appearance and duration of antibody response. As can be seen in Fig. 1, cross-reaction between analogues 1FV, RVG, GVE,
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TABLE III
Kinetic rate constants and equilibrium affinity constants of mAbs 4x8, 4x10, 4x11, 13x18, and 12x10 induced against IRGERA and IRGERA analogues

| mAbs | Antigen | $k_a$ ($x10^{-3}$) | $k_d$ ($x10^{-5}$) | $K_d$ ($x10^{-5}$) |
|------|---------|--------------------|--------------------|-------------------|
| 4x8 (IgG1) (anti-parent peptide) | Parent | 13 ± 0.2 | 15 ± 0.2 | 80 |
|     | IWR | 162 ± 0.6 | 166 ± 0.1 | 98 |
|     | RYG | 222 ± 0.5 | 163 ± 0.2 | 136 |
|     | GVE | 430 ± 0.5 | 61 ± 0.2 | 707 |
|     | EWR | nb | nb | nb |
|     | RFA | nb | nb | nb |
| 4x10 (IgG1) (anti-parent peptide) | Parent | 181 ± 0.8 | 180 ± 0.4 | 101 |
|     | IWR | 428 ± 0.6 | 53 ± 0.2 | 800 |
|     | RYG | 113 ± 0.6 | 169 ± 0.4 | 67 |
|     | GVE | 23 ± 0.9 | 172 ± 0.4 | 14 |
|     | EWR | nb | nb | nb |
|     | RFA | nb | nb | nb |
| 4x11 (IgG1) (anti-parent peptide) | Parent | 3 ± 0.2 | 100 ± 0.3 | 3 |
|     | IWR | 2600 ± 0.3 | 129 ± 0.3 | 2020 |
|     | RYG | 530 ± 0.5 | 50 ± 0.2 | 1060 |
|     | GVE | 540 ± 0.6 | 38 ± 0.2 | 1420 |
|     | EWR | 212 ± 0.3 | 82 ± 0.3 | 250 |
|     | RFA | 120 ± 0.4 | 63 ± 0.2 | 190 |
| 4x12 (IgG1) (anti-parent peptide) | Parent | 142 ± 0.5 | 210 ± 0.4 | 68 |
|     | IWR | nb | nb | nb |
|     | RYG | nb | nb | nb |
|     | GVE | nb | nb | nb |
|     | EWR | 52 ± 0.6 | 18 ± 0.4 | 293 |
|     | RFA | 1425 ± 0.5 | 66 ± 0.2 | 2170 |
| 13x18 (IgG2a) (anti-RI peptide) | RI | 830 ± 0.7 | 52 ± 0.2 | 1596 |
|     | IWR | 143 ± 0.3 | 65 ± 0.2 | 220 |
|     | RYG | 62 ± 0.2 | 33 ± 0.3 | 180 |
|     | GVE | 33 ± 0.1 | 76 ± 0.2 | 43 |
|     | EWR | 235 ± 0.5 | 130 ± 0.2 | 18 |
|     | RFA | 16 ± 0.5 | 120 ± 0.4 | 13 |
| 12x10 (IgG1) (anti-R peptide) | R | 328 ± 0.7 | 24 ± 0.2 | 1170 |
|     | IWR | nb | nb | nb |
|     | RYG | nb | nb | nb |
|     | GVE | nb | nb | nb |
|     | EWR | nb | nb | nb |
|     | RFA | nb | nb | nb |

a RI, retro-inverse peptide; R, retro-peptide.
b Association ($k_a$) and dissociation ($k_d$) rate constants are the mean values obtained in at least two independent experiments.
c nb, no binding.

and the peptide was found. Sometimes (see Fig. 1A, for example), cross-reacting antibodies appeared somewhat later than antibodies reacting with the homologous peptide analogue. It must be noted that, in mice immunized with analogue IWR (Fig. 1B), a higher antibody reaction was measured with analogues RΨG and GΨE than with the homologous peptide IΨR. Antisera directed to peptide analogues IΨR, RΨG, and GΨE (Fig. 1, B–D) reacted strongly with the cognate protein H3. In contrast, antibodies induced against analogues EΨR and RΨA (Fig. 1, E and F) recognized only the respective homologous peptides and did not react at all with heterologous peptides, nor with histone H3.

Resistance of Peptide Analogues to Trypsin—We have tested the resistance of peptide analogues to trypsin whose primary specificity is based upon positively charged lysine and arginine side chains. We used the enzyme covalently immobilized on nylon spheres and measured the residual capacity of proteolyzed peptides to compete with the parent peptide for the binding of mAb 4x11 in the BLAcore (1). mAb 4x11 was selected in this experiment because it reacts with all five intact reduced peptide bond peptide analogues (Table III). As shown in Fig. 2A, the parent peptide was rapidly digested under these conditions. Half of its antigenic activity was lost after 7 min and <10% activity remained after only 20 min. In contrast, after 4 h, only a slight loss of activity was observed; 71–97% of the initial antigenic activity remained upon incubation of the five reduced peptide bond peptide analogues with proteolytic beads.

The results were confirmed by HPLC. Only 16.8% of the parent peptide remained intact after 10 min of incubation with the proteolytic beads, whereas in the same conditions, 91.6–98.7% of the parent molecule (17–19).

DISCUSSION

The traditionally preferred route of delivery of pharmaceutical drugs is undoubtedly the oral route. However, oral delivery of peptides and proteins is generally hampered by the rapid hydrolysis of the peptide bonds by proteases of the digestive track and consequently other routes of administration have to be chosen. It is obvious that for chronic administration, intramuscular, intravenous, or subcutaneous injections are not desirable. Efforts have thus been developed to increase the stability of peptides and design pseudopeptides that keep their functions but are characterized by a high stability to proteolytic enzymes, which cannot cleave (or cleave much more slowly) a modified peptide bond (17). Pseudopeptides, also referred as amide bond surrogates, have been shown to convert endogenous neuropeptides or peptide hormones into more stable molecules, which could retain or even enhance the potency of the parent molecule (17–19). Recently, it has been shown in our group (1–4, 10, 20) and by others (5, 21, 22) that peptidomimetics could also be an useful alternative to natural peptides as ligands of immune system receptors and as immunogens. Guichard et al. (10, 20) and Hill...
et al. (21) used peptide analogues containing reduced peptide bonds and retro-inverso bonds to explore the requirements for peptide binding to class I and II MHC molecules, namely Kd and A2, and HLA DRB1*0101 and DRB1*0401, respectively. The use of such peptide analogues has not only a great interest for better understanding the conformational, topographical, and dynamic features of the MHC-peptide interaction, but it also allows us to develop peptide ligands that may be extremely relevant for immunomodulation. A series of recent studies have shown that slightly altered peptide ligands made by a single amino acid change could act as partial agonists or antagonists of cytolysis mediated by CD8+ T cell or of the CD4+ helper T cell response (23). Altered peptide ligands containing an isosteric replacement of the amide bond may also represent attractive candidates as components of such immunomodulatory strategy.

Another domain in which peptidomimetics may play a decisive role concerns vaccination. Because synthetic peptides provide a pathogen-free and relatively inexpensive means of delivering key antigenic determinants, particular attention has indeed been focused on their potential usage as components of synthetic vaccines. However, here again, a major limitation in their use is their instability as they are rapidly degraded by proteases. Injection of peptides generally takes place relatively far from their immunological receptors, requires transport in biological fluids and transit through several anatomical barriers, and thus is aimed at a long duration of action. In a previous work (4), we have shown that a retro-inverso analogue of a viral peptide induced a stronger and longer lasting antibody response than the homologous natural peptide. In the present work, we have investigated the antigenic and immunogenic properties of peptide analogues in which we have systematically introduced reduced peptide bonds between each amino acid residue at a time to determine the effect on their bioactivity.

Our data first confirm that reduced peptide bond pseudopeptides are remarkably more resistant to proteolysis than are natural peptides (Fig. 2). The antigenic activity of the five analogues that contain two arginine residues was almost the same after a 4-h incubation in the presence of trypsin, whereas
the antigenic properties of the natural peptide were lost after 20 min in the same conditions. The results were confirmed by following the hydrolysis of peptides by HPLC. The IRGERA peptide analogues potentially cleaved by trypsin after the two Arg residues, showed increased stability not only when the surrogate was introduced between Arg-Gly and Arg-Ala but also in other positions along the peptide sequence. Such a remote effect of single backbone modification on the stability of adjacent peptide bonds is well documented in peptide drug development (see Fauchère and Thurieau (17) and references therein). On the other hand, it is well established that, in the catalytic site of the enzymes of the chymotrypsin family, peptide backbone groups from P1 to P4 positions (according to the international nomenclature) of the substrate form an antiparallel β-sheet hydrogen bonding arrangement with the polar main chain atoms of the corresponding enzyme binding site (24). Consequently, the replacement of a peptide bond by the CH2–NH moiety at any position between P1 and P4 is expected to alter the substrate binding affinity since the methylene group has lost the potential of being part of hydrogen bonding interaction.

Second, as measured by the antigenic properties of the five analogues, the data show that, according to the antibody probes, the replacement of a particular natural peptide bond by a reduced peptide bond can alternatively have a positive effect (the strength of binding is higher), no effect, or a detrimental effect (Tables II and III). For example, antibodies from rabbits MEL, GIZ, and TRI as well as mAbs 4x8 and 4x10 (induced against the parent peptide or the cognate protein) did not react (or reacted significantly less well) with analogues EΨR and RΨA, which contain a reduced amide bond between R4-R5 and R5-R6. The deleterious effect may be explained as follows. At physiological pH, the reduced amide bond is protonated, and it has been shown by Marraud et al. (25) that the resulting ammonium link $\Psi(CH_2–NH_2^+)$ could stabilize a folded structure by acting as a strong proton donor. In addition, a potential hydrogen bonding point is lost in the replacement of the carbonyl by a methylene group. Thus, the expected alteration of the intramolecular hydrogen bond network and a conformational change of the C terminus in peptides EΨR and RΨA may explain the lack of reactivity of these analogues with some antibodies. This structural alteration in peptides EΨR and RΨA, however, seems to have no effect (or relatively less effect) on their recognition by other antibodies such as mAbs 4x11 and 13x18. Apparently, it even favors the binding of mAb 4x12 that in turn did not recognize analogues IΨR, RΨG, and GΨE.

By screening mAbs with the five reduced peptide bond analogues, a very interesting phenomenon could be observed: all four mAbs induced against the parent peptide (mAbs 4x8, 4x10, 4x11, and 4x12) were found to better react with certain analogues compared to the parent peptide. For example, the $K_a$ values of mAb 4x11 are increased 673, 353, 473, 83, and 63 times for analogues IΨR, RΨG, GΨE, EΨR, and RΨA, respectively, as compared with the $K_a$ value for the parent peptide. The $K_a$ value of mAb 4x12 for peptide analogue RΨA is increased 319 times. In general, it is due to a higher association constant ($k_a$); in some cases, both a higher $k_a$ and a slightly decreased $k_d$ explain the increased $K_a$ values. Such cases of heterospecificity (or heteroclitic effect) have already been described, for example with peptides in which particular amino acids have been substituted by another natural residue (26–28) or in which a natural bond has been replaced by a retro-inverso bond (2).

Third, the results reported here show that reduced peptide bond analogues are immunogenic. IgG antibodies were generally generated after three injections of the peptides covalently attached at the surface of small unilamellar vesicles including MPLA as adjuvant. We could not observe a longer lasting antibody response as shown previously in the case of retro-inverso analogues of peptide 141–159 of FMDV VP1 (4). It is possible that this lies to the particular feature of peptide IRGERA since likewise, we found no change in the kinetic of antibody response by introducing d-amino acid residues or retro-inverso peptide bonds in this peptide (1, 13). Antibodies raised against the parent peptide and analogues IΨR, RΨG, and GΨE were found to cross-react, and react also strongly with the cognate protein H3 (Fig. 1). In contrast, mice immunized against analogues EΨR and RΨA develop IgG antibodies, reacting only with the analogue used for immunization, further illustrating that the introduction of reduced peptide bonds between R4-R5 and R5-R6 changed the conformation of the resulting peptides sufficiently to drastically alter the antibody recognition. NMR studies of peptide-antibody complexes involving mAbs and the reduced peptide bond analogues described in this study are in progress and should unravel the structural basis of this type of interaction. On the other hand,
other reduced peptide bond analogues are being synthesized and will be investigated both as components of vaccine formulation and as immunomodulators in autoimmune models. The present study and other recent observations (1–5, 10, 20–22) open a new field of research in the development of stable peptidomimetics both for immunodiagnostic, synthetic vaccine design and immunotherapeutic purposes.

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REFERENCES
1. Guichard, G., Benkirane, N., Zeder-Lutz, G., Van Regenmortel, M. H. V., Briand, J.-P., and Muller, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9765–9769
2. Benkirane, N., Guichard, G., Van Regenmortel, M. H. V., Briand, J.-P., and Muller, S. (1995) J. Biol. Chem. 270, 11921–11926
3. Briand, J.-P., Guichard, G., Dumortier, H., and Muller, S. (1995) J. Biol. Chem. 270, 20686–20691
4. Muller, S., Guichard, G., Benkirane, N., Brown, F., Van Regenmortel, M. H. V., and Briand, J.-P. (1995) Pept. Res. 8, 138–144
5. Jameson, B. A., McDonnell, J. M., Martini, J. C., and Kornfeld, R. (1994) Nature 368, 744–746
6. Rammensee, H.-G. (1995) Curr. Opin. Immunol. 7, 85–96
7. Sloan-Lancaster, J., and Allen, P. M. (1995) Curr. Opin. Immunol. 7, 103–109
8. Jameson, B. A., and Kornfeld, R. (1995) J. Biol. Chem. 270, 20686–20691
9. Guichard, G., Calbo, S., Muller, S., Kourilsky, P., Briand, J.-P., and Abastado, J. P. (1995) J. Biol. Chem. 270, 20686–20691
10. Guichard, G., Benkirane, N., Graff, R., Muller, S., and Briand, J.-P. (1994) Pept. Res. 7, 308–321
11. Muller, S. (1988) Synthetic Polypeptides as Antigens (Van Regenmortel, M. H. V., Briand, J.-P., Muller, S., and Plaus, E., eds) pp 45–130, Elsevier Science Publishers B.V., Amsterdam
12. Friede, M., Muller, S., Briand, J.-P., Van Regenmortel, M. H. V., and Muller, S. (1993) J. Biol. Chem. 268, 26279–26285
13. Guichard, G., Calbo, S., Muller, S., Briand, J.-P., Van Regenmortel, M. H. V., and Muller, S. (1994) Mol. Immunol. 30, 539–547
14. Saunal, H., Karlsen, R., and Van Regenmortel M. H. V. (1996) in Immunochemistry 2. A Practical Approach (Johnstone, A. P., and Turner, M. W., eds) Oxford University Press, in press
15. Michalon, P., Couturier, R., Bender, K., Hecker, H., and Marion, C. (1993) Eur. J. Biochem. 216, 387–394
16. Fauchère, J. L., and Thureiau, C. (1992) Adv. Drug Res. 23, 128–154
17. Merrifield, R. B., Juvvadi, P., Andru, D., Ubach, J., Boman, A., and Boman, H. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3449–3453
18. Lehan, J. J., Kull, F. C. Jr., Landavazo, J. A., Stockstill, B., and Mcdermed, J. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 922–926
19. Guichard, G., Connan, P., Graff, R., Ostankovitch, M., Muller, S., Guillet, J.-G., Cheppin, J., and Briand, J.-P. (1996) J. Med. Chem. 39, 2030–2039
20. Hill, C. M., Liu, A., Marshall, K. W., Mayer, J., Jorgensen, B., Yuan, B., Cubbon, R. M., Nichols, E. A., Wicker L. S., and Rothbard, J. B. (1994) J. Immunol. 152, 2890–2898
21. Figueiredo, A., Ruvo, M., Cassani, G., and Fassina, G. (1995) J. Biol. Chem. 270, 30422–30427
22. Bondouli, I., Sakarellos, C., and Tzartos, S. J. (1993) Eur. J. Biochemistry 211, 227–234
23. Harper, M., Fassina, G., and Tzartos, S. J. (1993) J. Biol. Chem. 211, 97–108
24. Al Moudallal, Z., Briand, J.-P., and Van Regenmortel, M. H. V. (1982) EMBO J. 1, 1095–1101