Antigenicity and Immunogenicity of Modified Synthetic Peptides Containing D-Amino Acid Residues

ANTIBODIES TO A D-ENANTIOMER DO RECOGNIZE THE PARENT L-HEXAPEPTIDE AND RECIPROCALLY\(^*\)

(Received for publication, July 9, 1993, and in revised form, August 10, 1993)

Nadia Benkirane, Martin Friede\^{z}, Gilles Guichard, Jean-Paul Briand, Marc H. V. Van Regenmortel, and Sylviane Muller\(\ddagger\)

From the Institut de Biologie Mol\éculaire et Cellulaire, UPR 9021 Centre National de la Recherche Scientifique, 15 rue Descartes, 67000 Strasbourg, and the Laboratoire de Chimie Bioorganique, URA 1386 Centre National de la Recherche Scientifique, Facult\é de Pharmacie, 74 Route du Rhin, 67400 Illkirch, France

The effect of introducing d-amino acid residues in an hexapeptide was examined both at the antigenic and immunogenic levels. A series of d-analogues of the model peptide of sequence IRGERA corresponding to the COOH-terminal residues 130–135 of histone H3 were produced. Four analogues contained a single change of an L-residue by the corresponding enantiomer, one peptide contained two D-residues and another one contained only D-residues (d-enantiomer). A peptide analogue was also synthesized in which the 2 Arg residues were replaced by Lys residues. The parent peptide and peptide analogues were injected into mice after covalent coupling to small unilamellar liposomes containing monophosphoryl lipid A as adjuvant. The substitution of L-Arg\(^133\) to Lys or D-Arg was found to change neither the antigenic nor immunogenic properties of the resulting peptides. In contrast, the substitution of Glu\(^133\), Arg\(^134\), and Ala\(^135\) by the respective enantiomers drastically altered the antigenicity of the modified peptides. Each of the six D-analogues induced an immune response with an unusually high level of IgG3 antibodies. The D-enantiomer produced IgG3 antibodies which reacted with the homologous peptide as well as with the all L-peptide and the parent protein H3 in solution but not with analogues containing one or two D-residues only. IgG3 antibodies produced against the all L-peptide reacted with the free all D-peptide but not with the other analogues containing D-residues in position 133, 134, and 135.

Over the last decade, several candidate synthetic vaccines have been tested up to the level of in vivo protection trials. There has been an increasing tendency to design peptides endowed with a particular conformation and several peptides have been assessed as potential vaccines (Brown, 1990; Milich, 1990; Arnon and Van Regenmortel, 1992). Of particular interest is the possibility of improving the antibody response, in terms of specificity, isotype, and duration, by altering the presentation or composition of a peptide. To this end, peptide analogues containing D-amino acid residues or modified peptide bonds which exhibit a much higher resistance to proteolysis may be rendered, by virtue of their increased stability, more immunogenic than the native peptide. Such modifications have found many applications in the field of peptide-based drugs where increased stability has resulted in significantly increased biological activities (Fauchère and Thorieu, 1992). In contrast to studies dealing with pharmacological activities of peptides containing D-residues, very little has been published regarding the antigenic and immunogenic properties of such analogues (Geyser et al., 1986, 1987). In the present study, we have evaluated the influence of L- to d-amino acid substitutions on the antigenic and immunogenic properties of a model hexapeptide. A series of peptide D-analogues (several partially modified analogues and the mirror D-enantiomer; Wade et al., 1990) were tested in the hope of defining if changes in peptide structure lead to a longer duration of the immune response without impairing the capacity of modified peptides to induce antibodies cross-reacting with the cognate protein. In order to minimize any influence of the carrier, the parent peptide and analogues were injected into mice after coupling to small unilamellar liposomes containing monophosphoryl lipid A as adjuvant. The short peptide IRGERA which alone is not immunogenic (Frisch et al., 1991) was selected for this study. A short peptide was used since it seemed likely that with longer peptides, the presence of D-amino acids may also affect processing and presentation to MHC molecules (Milton et al., 1992; Jung, 1992) in addition to altering peptide stability.

**MATERIALS AND METHODS**

Peptides—Assembly of the protected peptide chains was carried out on a multichannel peptide synthesizer (Neimark and Briand, 1993) using the procedure described previously (Friede et al., 1992). Briefly, all amino acids were protected at the \(\alpha\)-amino position with the Fmoc group. The following side chain protecting groups were used: tert-butyl ester for Glu and 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl for Arg. Twenty-five \(\mu\)mol Fmoc-Ala or Fmoc-D-Ala 4-hydroxymethyl phenylmethyl polystyrene resin (Novabiochem, Switzerland) were placed in each reaction vessel. Fmoc amino acid deriv-
was added at an equimolar ratio to the MBP-PE and incubated at 37 °C for 1 h. After centrifugation at 10,000 rpm, the supernatant was removed, and the pellet was resuspended in 100 mM NaCl, 50 mM Tris-HCl, pH 7.4, and lyophilized.

Homogeneity of the crude peptides was assessed by analysis on a Novapak C18 column, 5 μm (3.9 × 150 mm) using a triethylammonium phosphate buffer system. Linear gradients were prepared with 100 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 10 mM NaAc, pH 5.0.

Preparation of Liposome-associated Peptides—Peptides were covalently coupled to preformed small unilamellar vesicles (SUV) containing 4-(p-maleimidophenyl)butyrylphosphatidylethanolamine (MBP-PE) (Friede et al., 1990). For immunization, monophosphoryl lipid A (MPLA) was incorporated into SUV in a preparatory step, followed by a final coupling reaction using the benzophenone species benzoylbenzoyl glycine which was added to the NH₂-terminal residue of the peptide IRGERA at the end of the synthesis (Muller, 1988). The yield of coupling was obtained by determining the amino acid composition of the final conjugate (Briand et al., 1993) or by determining spectrophotometrically the release of 2-thiopyridine from SPDP-derivated BSA on interaction with cysteine-containing peptides (Muller, 1988).

Preparation of Liposome-associated Peptides—Peptides were covalently coupled to preformed small unilamellar vesicles (SUV) containing 4-(p-maleimidophenyl)butyrylphosphatidylethanolamine (MBP-PE) (Friede et al., 1990). For immunization, monophosphoryl lipid A (MPLA) was incorporated into SUV in a preparatory step, followed by a final coupling reaction using the benzophenone species benzoylbenzoyl glycine which was added to the NH₂-terminal residue of the peptide IRGERA at the end of the synthesis (Muller, 1988). The yield of coupling was obtained by determining the amino acid composition of the final conjugate (Briand et al., 1993) or by determining spectrophotometrically the release of 2-thiopyridine from SPDP-derivated BSA on interaction with cysteine-containing peptides (Muller, 1988).

Preparation of Liposome-associated Peptides—Peptides were covalently coupled to preformed small unilamellar vesicles (SUV) containing 4-(p-maleimidophenyl)butyrylphosphatidylethanolamine (MBP-PE) (Friede et al., 1990). For immunization, monophosphoryl lipid A (MPLA) was incorporated into SUV in a preparatory step, followed by a final coupling reaction using the benzophenone species benzoylbenzoyl glycine which was added to the NH₂-terminal residue of the peptide IRGERA at the end of the synthesis (Muller, 1988). The yield of coupling was obtained by determining the amino acid composition of the final conjugate (Briand et al., 1993) or by determining spectrophotometrically the release of 2-thiopyridine from SPDP-derivated BSA on interaction with cysteine-containing peptides (Muller, 1988).

Preparation of Liposome-associated Peptides—Peptides were covalently coupled to preformed small unilamellar vesicles (SUV) containing 4-(p-maleimidophenyl)butyrylphosphatidylethanolamine (MBP-PE) (Friede et al., 1990). For immunization, monophosphoryl lipid A (MPLA) was incorporated into SUV in a preparatory step, followed by a final coupling reaction using the benzophenone species benzoylbenzoyl glycine which was added to the NH₂-terminal residue of the peptide IRGERA at the end of the synthesis (Muller, 1988). The yield of coupling was obtained by determining the amino acid composition of the final conjugate (Briand et al., 1993) or by determining spectrophotometrically the release of 2-thiopyridine from SPDP-derivated BSA on interaction with cysteine-containing peptides (Muller, 1988).

Preparation of Liposome-associated Peptides—Peptides were covalently coupled to preformed small unilamellar vesicles (SUV) containing 4-(p-maleimidophenyl)butyrylphosphatidylethanolamine (MBP-PE) (Friede et al., 1990). For immunization, monophosphoryl lipid A (MPLA) was incorporated into SUV in a preparatory step, followed by a final coupling reaction using the benzophenone species benzoylbenzoyl glycine which was added to the NH₂-terminal residue of the peptide IRGERA at the end of the synthesis (Muller, 1988). The yield of coupling was obtained by determining the amino acid composition of the final conjugate (Briand et al., 1993) or by determining spectrophotometrically the release of 2-thiopyridine from SPDP-derivated BSA on interaction with cysteine-containing peptides (Muller, 1988).

RESULTS

Synthetic Peptides—Eleven peptides were used in this study (Table I). Peptides 1–4 correspond to the natural sequence of the COOH-terminal portion of histone H3 (IRGERA). This region contains a major epitope of H3 which has been extensively studied in this laboratory (Muller et al., 1982; Frisch et al., 1991; Briand et al., 1992; Friede et al., 1993). In order to enhance the accessibility of the residues in the IRGERA and GERA peptides bound to a carrier, two additional residues of Gly were added to their NH₂-terminal end (peptides 1 and 4–11). A Cys residue was added at the NH₂-terminus of these peptides to allow selective conjugation of the peptides to BSA and liposomes via reaction with maleimide groups present on the activated carriers. In peptide 5, Arg9 and Arg14 were replaced by Lys residues and in peptides 6–11, D-amino acid residues replaced certain L-amino acid residues as indicated in Table I. The replacement by a D-optical isomer is not applicable to the symmetrical Gly13. The degree of purity of peptides, as assessed by high performance liquid chromatography, was at least 85%. In CD measurements a longer peptide (residues 113–135) was introduced as control.

Structure of Peptide IRGERA and Peptide Analogues—In absence of structural data, attempts to compare the structures

| Table I | Sequences of the COOH-terminal peptides of histone H3 and analogues |
|---------|---------------------------------------------------------------|
| Peptides | Sequence |
| 1        | C G G G E R A A |
| 2        | R G E R A A |
| 3        | B B G G G G G E R A A |
| 4        | G G G G G G G G G G E R A A |
| 5        | G G G G G G G G G G E R A A |
| 6        | C G G G G G G G G G G E R A A |
| 7        | C G G G G G G G G G G E R A A |
| 8        | C G G G G G G G G G G E R A A |
| 9        | C G G G G G G G G G G E R A A |
| 10       | C G G G G G G G G G G E R A A |
| 11       | C G G G G G G G G G G E R A A |

1 The abbreviations used are: TFE, trifluoroethanol; BSA, bovine serum albumin; SPDP, N-succinimidyl 3-[2-pyridyl dithiolpropionate (SPDP); T), antiserum diluted in PBS-T containing 10 mg/ml BSA was added to the activated carriers. In peptide 5, Arg9 and Arg14 were replaced by Lys residues and in peptides 6–11, D-amino acid residues replaced certain L-amino acid residues as indicated in Table I. The replacement by a D-optical isomer is not applicable to the symmetrical Gly13. The degree of purity of peptides, as assessed by high performance liquid chromatography, was at least 85%. In CD measurements a longer peptide (residues 113–135) was introduced as control.
adopted by the different peptide analogues by molecular modeling using calculations of energy minimization were unsuccessful. No preferential conformation was observed and thus modeled structures were of very low reliability (data not shown). The crystal structure of the core histone octamer has been determined at 3.1-A resolution (Arents et al., 1991). In the three-dimensional structure the residues in region 120–132 of H3 form an a-helix, while the 3 residues 133–135 are outside the helix. CD spectra show that in 100% TFE, the percent helix content in peptide 118–135 may be estimated as 30% (Fig. 1). Helical conformation is no longer observed in peptide 4 and a negative ellipticity at 198 nm is found indicating an unordered form. Although CD spectra of peptides in TFE need to be interpreted with great circumspection, it is interesting to observe that spectra obtained with peptides 4 and 11 are roughly symmetrical.

Recognition in ELISA of IRGERA Analogues by Rabbit and Mouse Antibodies Induced against the Parent Peptide IRGERA and H3—Three antisera raised against the peptide IRGERA (rabbits Mel and Giz) and histone H3 (rabbit Tri) were first tested for their ability to react with the various peptides shown in Table I. These tests were performed in a competitive ELISA using H3 as antigen for coating plates and the different peptides as inhibitors. As shown in Fig. 2 and Table II, the binding of anti-H3 antibodies to H3 was strongly inhibited by peptides 4, 5, and 6, less well by the shorter peptide 1 and not at all by peptides 7–11. Very little difference was seen when rabbit antibodies against peptide IRGERA instead of rabbit antibodies against the cognate protein H3 were used except for peptide 8 which inhibited slightly the interaction of anti-IRGERA antibodies with H3.

Antisera against peptide 4 were also raised in BALB/c mice by a series of intraperitoneal injections of the peptide coupled at the surface of SUV containing MPLA as adjuvant (Friede et al., 1993). When the antisera were tested in the competitive assay using H3 as antigen and the different peptides as inhibitors, very similar results to those described above with rabbit antisera were found (Table II). Antibodies raised against peptide 4 reacted also strongly with H3 in solution (see below).

The cross-reaction of antibodies against peptide 4 with the various peptide analogues and with H3 was also studied in a direct ELISA format in which H3 and the different peptide analogues conjugated to BSA by means of SPDP were used for coating plates. The conditions of coupling were controlled in such a way that all BSA-peptide conjugates had the same peptide to carrier molar ratio of 10. As shown in Table III, mouse antibodies against peptide 4 reacted well with homologous peptide 4 (see also Fig. 3A) as well as with the peptide analogues 5 and 6. They also reacted strongly with the parent protein H3 (Fig. 3F). In contrast they did not bind to peptides 7–11.

From the results obtained with rabbit and mouse antisera, it can be concluded that the replacement of Argx3 and Argx34 by Lys residues (peptide 5) had no effect on antibody recognition. Likewise, the replacement in peptide 6 of Argx34 by D-Arg had no detectable effect on the binding of anti-H3 and anti-IRGERA antibodies. In contrast, the replacement in peptide 4 of L-residues 133, 134, and 135 by the respective D-isomers (peptides 7–9) dramatically altered the recognition of the peptides by anti-H3 and anti-IRGERA antibodies. Likewise, peptides 10 and 11 containing 2 D-Arg residues in position 131 and 134 and 5 D-residues in position 130–135, respectively, were recognized neither by H3 nor by IRGERA antibodies.

Mouse Antibodies to IRGERA Analogues—Groups of four BALB/c mice were injected with the various peptide analogues conjugated to liposomes. An IgM response could be generally demonstrated by ELISA in bleeding 1 which slowly decreased from bleeding 2 onward and was no longer detectable after bleeding 5 (data not shown). IgG responses to the different peptides conjugated to BSA by means of SPDP are shown in Fig. 3 (open squares). IgG antibodies were generally detected from bleeding 2 onward and, depending on the peptide used as immunoconjugate, their activity decreased from bleeding 5 (47 days after the last injection) to 7 (89 days after the last injection). A fairly low response was found in mice immunized with peptides 7 and 11 (Fig. 3, D and H). The antibody response measured by ELISA was very similar in the individual animals of each group.

Ability of Mouse Antibodies Induced against IRGERA Analogues to Cross-react with Histone H3 and Various Peptide Analogues—The capacity of mouse antibodies induced with IRGERA analogues coupled to liposomes to recognize histone H3, parent peptide 4, and other IRGERA analogues was studied in different ELISA formats. The reactivity of mouse antisera was first studied in a direct ELISA in which the different antigens were adsorbed on microroller plates (Table III, Fig. 3, open squares). The reactivity of antibodies against peptide analogues containing Lys residues at positions 131 and 134 (peptide 5) or a D-Arg residue at position 131 (peptide 6) was very similar to that of antibodies against peptide IRGERA (peptide 4). These antibodies reacted with peptides 1, 4, 5, and 6 and with the cognate protein H3 but not with peptides 7–11. In contrast, antibodies induced against peptides 7–11 recognized only the respective homologous peptides and did not react with heterologous peptides nor with H3.

The reactivity of the mouse antibodies was also tested in a competitive binding assay with free peptides in solution to determine if the free peptides were recognized as well as the peptide conjugates. The results presented in Table IV show the molar excesses of the various peptides required to inhibit 50% of the binding of anti-peptide antibodies and the respective homologous peptides. In agreement with the results obtained with immobilized conjugated peptides, peptides 4, 5, and 6 strongly inhibited the binding of antibodies against these peptides, but not that of antibodies against peptides 7–11. As also observed in direct ELISA, only the homologous peptides inhibited the reaction between antibodies to peptides 7–11 and their respective peptides. H3 in solution inhibited

---

2 P. Orlewski and M-T. Cung, Nancy, France.

3 E. M. Moudrianakis, personal communication.
the binding of antibodies induced against peptides 1, 4, 5, and 6, but not that of antibodies against peptides 7-11, to their respective homologous peptides coated as BSA-conjugates (Table V).

Overall, when the results obtained in the direct and competitive ELISA tests were compared, the only difference which was observed concerned peptide 1. In solution this short peptide was only capable of inhibiting the binding between anti-peptide 1 antibodies and peptide 1 and was unable to compete with IRGERA analogues (Table IV). In a direct binding assay, this peptide coated as a BSA-conjugate was recognized by antibodies induced against peptides 4, 5, and 6 while anti-peptide 1 antibodies recognized conjugated peptides 4, 5, and 6 (Table III).

The binding of H3 by antibodies induced against peptides 4, 5, and 6 was strongly inhibited by the homologous peptides but not by peptide 1 and peptides 7-11 (data not shown). The binding of H3 by antibodies to peptide 1 was only inhibited by peptide 1.

**Analysis of the Isotypes of Antibodies Raised to IRGera and IRGera Analogues Associated to SUV Containing MPLA**—The antibodies raised in BALB/c mice to the various peptides covalently attached to SUV were essentially of the IgG type. Antibodies of IgM isotype which were initially present gradually disappeared during the course of immunization, and no IgA antibodies were detected. Antibodies of all IgG subclasses were present in the various antipeptide sera except in antisera to peptide 11 which contained mainly IgG3 antibodies (Fig. 3H). The IgG3 subclass (Fig. 3, closed squares) was also prevalent in antisera to peptides 7 and 9 (Fig. 3, D and F).
D-Analogues of Antigenic Peptides

26283

n
C
E
v

\[ \text{FIG. 3. Test in ELISA of the antibody response to the eight peptides 4-11 injected as SUV-coupled peptides in BALB/c mice. Antisera were diluted 1:500 and allowed to react with the homologous peptides (A-H) or with H3 (I-P). Anti-mouse IgG (H+L) conjugate (C) and anti-mouse IgG conjugate (D) were diluted 1:5,000. Absorbance values were measured at 450 nm. The results represent the average absorbance values obtained in each group of mice (n = 4).} \]

| Peptides used as inhibitor | Molar excess of inhibitor peptide required to inhibit 50% of the binding between anti-peptide antibodies and homologous antigens |
|----------------------------|------------------------------------------------------------------------------------------------------------------|
| 1  | 10  | -  | 4  | -  | 5  | -  | 6  | -  | 7  | -  | 8  | -  | 9  | -  | 10 | -  | 11 |
| 4  | -  | 30  | 40  | 50  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 5  | -  | 20  | 10  | 10  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 6  | -  | 20  | 10  | 10  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 7  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 8  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 9  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 10 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 11 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

* - no detectable cross-reactivity (up to 125 μg/ml peptide).

By using in ELISA a conjugate specifically directed toward mouse IgG3, it was possible to detect a particular subpopulation of antibodies of the IgG3 isotype which was produced generally later during the course of immunization. This reactivity was not detected when anti-globulin conjugate was a mouse IgG (H+L) second antibody, presumably because the conjugate contained too few anti-IgG3 antibodies.

**TABLE IV**

Recognition in competitive ELISA of IRGERA and IRGERA analogues by mouse antibodies induced against homologous and analogue peptides

Microtiter plates were coated with 2 μM peptide conjugated to BSA by means of SPDP (carrier peptide molar ratio 1:10) and allowed to react with mouse antisera raised against the homologous peptide (serum dilution 1:500) and preincubated with the various peptides used as inhibitors. Anti mouse IgG peroxidase conjugate was diluted 1:5,000.

When the specificity of the IgG3 antibodies induced against the peptides 6-11 was tested in direct and competitive ELISA, it was found that these antibodies reacted in the same way with the various antigens as the other IgG subclasses except in the following two cases: 1) the IgG3 anti-peptide antibodies, especially those induced against peptides 7-11, reacted well and, in some cases, strongly with histone H3 (Fig. 3, L-P). 2) free peptides 4 and 11 but not the other peptide analogues inhibited the binding between H3 and IgG3 antibodies induced against peptide 11 (Fig. 4A). In reciprocal tests, free peptides 4 and 11 but not the other peptide analogues, inhib-
D-Analogues of Antigenic Peptides

Fig. 4. Inhibition of the ELISA reaction between H3 (100 ng/ml) and IgG3 antibodies directed to peptide 11 (A) or to peptide 11 (B) by increasing concentrations of peptides 4–11. Mouse antisera were diluted 1:500 and anti-mouse IgG3 second antibody 1:5,000. Absorbance values were measured at 450 nm. The molar excesses of peptides 4 and 11 over H3 required to inhibit 50% of the antibody binding were about 300 in both cases.

were the peptide analogues containing D-residues at positions 133, 134, and 135 recognized by antibodies against H3 or peptides 4, 5, and 6. The various partially D-analogues also differ significantly among themselves since they were not recognized by antibodies induced against the non-homologous peptide analogues either in the direct or in the competition ELISA format where peptides are in the free form.

Regarding the immunogenicity of the D-analogues, we found that each of the six D-analogues when coupled at the surface of SUV containing MPLA induced an immune response. The response, as revealed with anti-total IgG conjugate (which very likely detects IgG1, IgG2a, and IgG2b but not IgG3) was somewhat lower in the case of peptides 7 and 11 and, in general, of shorter duration in the case of all peptides containing D-residues. In contrast, IgG3 antibodies appeared later in the serum of immunized mice, reached a maximum in bleedings 4–5 and, in some cases (peptides 7, 9, and 11), remained at a significant level in bleeding 7, i.e. 70 days after the last injection. Thus the progression of IgG1, 2a, and 2b antibodies and that of IgG3 antibodies did not follow the same pattern. Furthermore, antibodies of the IgG1, 2a, and 2b subclasses were produced at a low level in response to injection of the D-enantiomer which initially led us to believe that this peptide was less immunogenic than the others.

Overall, the results lead to some important conclusions. First, it seems that the presence of IgG3 antibodies in the immunized mice can be missed when an anti-mouse IgG (H+L) peroxidase conjugate is used. It is important, therefore, to determine which subclasses of IgG are actually detected by the indicator immunoconjugate and to use reagents that reveal all antibodies with the same efficacy.

Second, the high IgG3 response in all mice immunized with the various peptides coupled to the surface of SUV is particularly striking. Although we cannot directly compare the levels of reactivity of the antibodies of different subclasses because the anti-mouse conjugates used in our ELISA procedure were different and that one arbitrary working dilution of 1:5,000 was chosen, the IgG3 response detected in immunized mice was significantly higher than usual. This finding may result in part from the fact that we have used small liposomes with surface-bound peptide and not a carrier protein for presenting peptides, and that MPLA, and not Freund's adjuvant, was used as adjuvant. Interestingly it has been reported that mouse IgG3 subclass antibodies predominate in immunized mice as well as in the immune response to protein antigens linked to the surface of liposomes while they constitute a small component of the humoral response to encapsulated protein (Thérien et al., 1991) which is suggestive of a T-independent B-cell activation (McKearn et al., 1982; Snapper and Mond, 1993).

Third, when the specificity of antibody response is considered, the antibody population of the IgG1, 2a, and 2b subclasses appears very specific in the sense that the antibodies of these subclasses induced against peptides 7, 8, 9, and 10, for instance, recognized only the homologous analogue peptides and not the other partially modified D-analogues or the cognate protein H3. In contrast, the IgG3 population contains antibodies able to react both with the respective homologous peptides and with H3 on the plastic. Most interesting was the fact that the parent peptide as well as the protein H3, both in the free form, were also recognized by anti-all D-peptide IgG3 antibodies. Reciprocally, IgG3 antibodies induced against the parent peptide did recognize the homologous peptide and H3 as well as the free all D-peptide. This cross-reactivity was only found between the parent peptide and the D-enantiomer (mirror image) but not when a single

DISCUSSION

Among the factors thought to affect the duration of the immune response, persistence of the antigen in an appropriate location is considered to play an important role (Gray and Skarvall, 1988). Thus one of the major problems in developing synthetic vaccines is to enhance the half-life of the peptides and increase the probability that they will interact with cells of the immune system, in particular with dendritic cells. In this study, we have used the liposome-peptide model developed previously (Friede et al., 1993) and explored the effect of replacing L-amino acid residues by the corresponding D-amino acids on both the duration and specificity of the immune response. To the best of our knowledge this is the first detailed investigation of the influence of enantiomeric substitutions on the immunogenicity of peptides.

As measured by the antigenic properties of the seven analogues produced in this study, the data suggest that neither the substitution of Arg\[^{235}\] and Arg\[^{254}\] by Lys residues nor the replacement of Arg\[^{235}\] by a D-Arg changed the conformation of the resulting peptides sufficiently to alter the binding of rabbit and mouse antibodies induced against H3 or against peptides 4, 5, and 6. In contrast, the substitution of Glu\[^{132}\], Arg\[^{235}\], and Ala\[^{138}\] by the respective enantiomers (peptides 7, 8, and 9) drastically altered the antigenicity of the modified peptides. In none of the presentations (either coupled to BSA by means of SPDP or free in solution in inhibition assays)
D-amino acid was introduced in the sequence of the hexapeptide. The common structure between a peptide and its mirror image is the peptide backbone (Guptasarma, 1992). Accordingly, the results suggest that the IgG3 antibodies recognize the backbone of the peptides 4 and 11 rather than the side chains. In this regard, it is noticeable that in particular cross-reactivity with IgG3 antibodies to peptide 11, the parent peptide 4 and the peptide 5 containing 2 lysine residues in replacement of arginine residues were not antigenically equivalent.

It is well established that all L-amino acid polymers make right-handed α-helices whereas homopolymers of D-amino acid residues form left-handed ones. Therefore, the fact that IgG3 antibodies induced against the D-enantiomer reacted with the parent protein H3 and that IgG3 antibodies induced against the all L-peptide reacted with the free D-enantiomer points to new possibilities of manipulating peptide antibody responses. Furthermore, the fact that it is only the IgG3 subclass that exhibits this cross-reactivity suggests that this class of antibody may bind to antigen in a unique manner possibly allowing a self-association which yields more effective binding, for example to the surface of bacteria (Greenspan and Cooper, 1992). NMR studies of peptide-antibody complexes involving these analogues and monoclonal antibodies (Cung et al., 1991) may unravel the structural basis of this particular type of interaction.

Acknowledgments—We are grateful to Drs. E. M. Moudrianakis (Baltimore) and E. Weisshof (Strasbourg) for helpful advice, to Drs. P. Orlewski and M-T. Cung (Nancy) for attempts of molecular modeling, to Dr. M. John (Strasbourg) for help with CD measurements and to G. Sommermeyer for valuable technical assistance.

REFERENCES
Arenta, G., Burlingame, R. F., Wang, B-C., Love, W. E., and Moudrianakis, E. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10148-10152

Arnon, R., and Van Regenmortel, M. H. V. (1992) FASEB J. 6, 3285-3287
Briand, J. P., Muller, S., and Van Regenmortel, M. H. V. (1985) J. Immunol. Methods 76, 59-69
Briand, J. P., Barin, C., Van Regenmortel, M. H. V., and Muller, S. (1992) J. Immunol. Methods 166, 255-265
Brown, F. (1990) Semin. Viroi. 1, 67-74
Butler, J. E., Li, L., Nessler, R., Joshi, K. S., Suter, M., Rosenberg, B., Chang, J., Brown, W. R., and Cantarero, L. A. (1992) J. Immunol. Methods 150, 77-90
Cung, M. T., Demange, P., Marraud, M., Taikaria, V., Sakarellos, C., Papadoulis, I., Kokta, A., and Tsartos, S. (1991) Biopolymers 31, 769-776
Fanichel, J. L., and Thuret, C. (1992) Adv. Drug. Res. 29, 127-159
Friede, M., Denesy, S., Neimark, J., Kieffer, S., Gaussepoil, H., and Briand, J. F. (1992) Peptide Res. 5, 145-147
Friede, M., Muller, S., Briand, J. F., Van Regenmortel, M. H. V., and Schuber, F. (1993) Mol. Immunol. 30, 539-547
Frach, B., Muller, S., Briand, J. F., Van Regenmortel, M. H. V., and Schuber, F. (1991) Eur. J. Immunol. 21, 180-193
Geyser, M. H., Rodda, S. J., and Mason, T. J. (1986) Mol. Immunol. 24, 709-715
Geyser, H. M., Macfarlan, R., Rodda, S. J., Tribbick, G., Mason, T. J., and Shorth, P. (1987) in Towards Better Carbohydrate Vaccines (Bell, R., and Torregiani, G., eds) pp. 103-113, John Wiley and Sons, Chichester
Gray, D., and Biarrall, H. (1988) Nature 336, 70-73
Greenspan, N. S., and Cooper, L. J. N. (1995) Immunol. Today 16, 164-168
Guptasarma, P. (1992) FEBS Lett. 310, 205-210
Hudson, D. (1968) J. Org. Chem. 33, 617-624
Jung, G. (1992) Angeu. Chem. Int. Ed. Engl. 31, 1457-1459
Kung, D., Fields, C., and Fields, G. (1990) Int. J. Peptide Protein Res. 36, 255-266
McKee, J. P., Paslay, J. W., Slack, J., Baum, C., and Davey, J. M. (1992) Immunol. Rev. 124, 5-28
Millich, D. R. (1996) Semin. Immunol. 2, 307-315
Milton, R. C., de L, Milton, S. C. F., and Kent, S. B. H. (1992) Science 256, 1448-1448
Muller, S. (1988) in Synthetic Polypeptides as Antigens (Van Regenmortel, M. H. V., Briand, J. F., Muller, S., and Plasue, S., eds) pp. 159-175, Elsevier, Amsterdam
Muller, S., Himmelsbach, K., and Van Regenmortel, M. H. V. (1982) EMBO J. 412-429
Muller, S., and Van Regenmortel, M. H. V. (1998) Methods Enzymol. 170, 251-263
Neimark, J., and Briand, J. P. (1993) Peptide Res. 6, 219-228
Snapper, C. M., and Mond, J. J. (1989) Immunol. Today 14, 15-17
Sorensen, K., and Brodb Heck, U. (1986) J. Immunol. Methods 95, 291-293
Therien, H. M., Shahum, E., and Pertin, A. (1991) Cell. Immunol. 136, 402-413
Wade, D., Boman A., Whalin, B., Drain, C. M., Andrew, D., Boman, H. G., and Merrifield, R. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4781-4785