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Helix Stabilization in the C-Terminal Peptide of Chicken Riboflavin Carrier Protein Enhances Immunogenicity and Prolongs Contraceptive Potential as an Epitope-Based Vaccine in Female Rats

Sarada Subramanian,* Anjali A. Karande,† and P. Radhakantha Adiga*,[1]
**Department of Molecular Reproduction, Development, & Genetics and †Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India*

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Earlier investigations have shown that (a) antibodies against a carrier-coupled 20-residue synthetic peptide (C-20), ^{200}HACQKKLLKFEALQQEEGEE^{219}, corresponding to the C-terminal partially helical sequence of chicken riboflavin carrier protein (RCP; 219 AA) curtail pregnancy in mammals and (b) helix stabilization by introducing appropriately spaced salt bridges in the flanking sequences of its B-cell epitopic structure enhances RCP antigenicity to peptide antibodies. Among such engineered C-20 analogs, HE-20 (HAEQKKLLKFEALEQEKGKE) exhibited maximum helical propensity. Since C-20 per se, i.e., without carrier conjugation, elicits RCP-reactive neutralizing antibodies in rodents, we mapped its T-cell epitope which overlaps its B-cell epitope, both of which remain unmodified in HE-20. Comparative evaluation of immunogenicity of the two epitope-based peptide vaccines showed that HE-20 was far superior to C-20 in generating RCP-reactive antibodies in terms of both affinity and titer. With regard to bioefficacy, passive immunoneutralization of RCP in pregnant rats by administering purified IgG from either of the antipeptide sera terminated pregnancy. Similarly, active immunization of fertile female rats with the individual peptide analogs curtailed pregnancy. However, HE-20 was more efficient in eliciting higher affinity, longer-lasting, RCP-crossreactive antibodies with consequently more prolonged immunocontraceptive efficacy.

Key Words: **antigenicity; contraception; helix stabilization; immunogenicity; riboflavin carrier protein; synthetic peptides.**

The riboflavin-carrier (RCP) or -binding protein (RfBP; 219 AA), an estrogen-inducible phosphoglycoprotein, is obligatory for transporting riboflavin from the maternal system to the developing oocyte in the egg-laying chickens and thus plays a definitive role in embryonic development (1). RCP is evolutionarily conserved in a wide variety of species ranging from lower vertebrates to mammals including humans (2–5). Earlier studies have unequivocally established the importance of this carrier protein to support embryonic growth since immunoneutralization of endogenous maternal RCP with antibodies to the heteroantigen viz. chicken RCP leads to early embryonic demise and hence pregnancy termination both in rodents (6) and subhuman primates (7). Passive immunization of mice with a mouse monoclonal antibody (mAb) 6B2C12, directed toward the C-terminal fragment of RCP induced high rates of embryonic resorption (8). The core sequence recognized by this mAb has been mapped to ^{203}QKKLLKFEAL^{212} of the protein (9). A 20-amino-acid synthetic peptide (C-20) corresponding to residues 200–219 of chicken RCP (Fig. 1) coupled to diphtheria toxoid (DT) as the carrier is effective in inducing antipeptide and anti-RCP antibodies which caused early embryonic resorption in pregnant mice and rats in experiments involving either active or passive immunization (9). Conformational studies on the synthetic C-20 peptide suggested the involvement of helical structure in determining the peptide antigenicity (10). Since incorporation of specific stereochemical constraints into an antigenic peptide that stabilize the conformational states recognized by antibodies improves the immunological characteristics of such engineered peptides (11–14), a molecular design approach involving stepwise introduction of sequence changes that facilitate incremental stabilization of a native-like secondary structure was adopted (15). This strategy resulted in peptide HE-20 (Fig. 1) with highest helical potential among engineered peptides following incorporation of $i \rightarrow i + 3/i + 4$ salt bridges involving lysine

[1] To whom correspondence and reprint requests should be addressed. Fax: 91 80 3600999. E-mail: adiga@mrdg.iisc.ernet.in.

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Sequence	Nomenclature
HACQKKLLKFEALQQEEGEE	C-20
HAEQKKLLKFEALEQEKGKE	HE-20

FIG. 1. Sequence of the peptides used in the present study. The substituted amino acids are underlined in the modified peptidyl sequence.

and glutamic acid pairs in the flanking sequences at both the N- and C-terminal to the core B-cell epitopic sequence. This design also abolishes the clustering of negative charges near the C-terminus, a feature which may further enhance helix stability. In earlier studies designed to probe possible correlation between helicogenic potential of the engineered peptides and immunogenicity, we used these peptides coupled to carrier protein (DT) as immunogens (15) and found that anti-peptide antibodies so elicited interacted with native RCP with relative affinities proportional to their helical propensity. With recent evidence in our laboratory (16) that these peptides per se function as minivaccines to elicit anti-peptide and anti-RCP antibodies, it was of interest to map the T-cell epitopic sequence contained therein, compare immunogenic potential of the structured (HE-20 vis-a-vis the native C-20) peptide and to examine the efficacy of active immunization with these peptides in terms of contraceptive potential in adult female rats.

MATERIALS AND METHODS

Materials. *N*-α Fmoc amino acid pentafluorophenyl esters were purchased from Nova Biochem (UK). Dimethyl formamide, piperidine, *t*-amyl alcohol, 1-hydroxy benzotriazole, trifluoroacetic acid, and Ultrosyn KA resin (0.1 m equivalent/g) were procured from Pharmacia, Sweden. Freund's complete and incomplete adjuvants (FCA and FIA, respectively), alkaline phosphatase-conjugated goat anti-mouse, goat anti-rat, and goat anti-rabbit IgG, *p*-nitrophenyl phosphate, and Tween 20 were obtained from Sigma Chemicals Co. (U.S.A.). The chicken RCP was purified to homogeneity from the egg white as described previously (17). The mAb 6B2C12 available in the laboratory was raised against chicken RCP and characterized earlier (8). The peptides were synthesized by solid-phase method using F-moc chemistry as described (15). The peptides were synthesized on a LKB-Biolynx 4175 semiautomatic peptide synthesizer and their purity checked by reverse phase HPLC on a Lichrosorb C18 column (4×250 mm, 5 μ), and the sequence analyzed on a gas-phase sequenator (Shimadzu, Model PSQ1).

Animals. Wistar rats and albino rabbits used for immunization were procured from the Institute's Central Animal Facility. These animals were fed pelleted diet and water *ad libitum*. They were exposed to 12-h light/12-h dark schedule under controlled conditions of humidity and ambient temperature.

T-cell proliferation assay. Lymphocyte stimulation assays were performed as described elsewhere (18). Briefly, 3-month-old rats were injected subcutaneously with 100 μg of each of the peptides emulsified in FCA, in the hind foot pads. After 10 days, popliteal lymph nodes were aseptically dissected and single cell suspensions prepared. About 4×10^5 lymph node cells were cultured in 200 μl of RPMI 1640 supplemented with 10% fetal calf serum and 2 mM glutamine. Indicated amounts of the individual peptides were added as antigen source. Phytohemagglutinin (5 μl/well) was used as a positive control. The assay was performed in triplicate in 96-well microtiter plates. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. After an 18-h pulse with 1 μCi [^{3}H]thymidine per well at the end of a 4 day incubation period, the proliferative response was measured on harvested cells in a β-scintillation counter. At least three repetitions of the assay were performed with equivalent results. Data are presented as T-cell proliferative indices calculated as the [^{3}H]thymidine incorporation by cells cultured with the peptide relative to the [^{3}H]thymidine incorporation by cells cultured in the absence of the peptide.

Production of anti-peptide antibodies. Individual peptides emulsified in FCA were injected into rabbits at a dose of 1 mg/animal for primary injection. For subsequent boosters at 3-week intervals, 500 μg of peptide emulsified in FIA was administered subcutaneously. After three boosters, the rabbits were bled, sera collected and stored in aliquots at −20°C until used.

Characterization of the peptides antisera. The immune recognition patterns of these antisera toward the corresponding free peptides as well as the native RCP were determined by direct ELISA (8). Briefly, either 2 μg of peptide or 1 μg of chicken RCP was coated in a high binding ELISA plate. After blocking the unoccupied sites with 0.3% (w/v) BSA, these wells were probed with serially double diluted antisera. The antigen–antibody interaction was visualized by employing alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-rat IgG and *p*-nitrophenyl phosphate as the chromogen. Color development was monitored at 405 nm. The affinity of these peptide antisera to the corresponding parent peptide as well as the native RCP was determined by inhibition ELISA (19).

Purification of IgG. Peptide antisera were dialyzed against 0.1 M phosphate, pH 8.0, and loaded onto protein A-Sepharose affinity column pre-equilibrated with the same buffer. The bound IgG were eluted with 0.1 M glycine, pH 3.0. Fractions (1 ml) were collected into tubes containing 20 μl of 2 M Tris (to immediately neutralize the pH). Fractions showing absorbance at 280 nm were pooled, dialyzed against PBS and checked for binding to the corresponding peptides and the native RCP by ELISA.

Immunoneutralization studies. For passive immunization, regularly cycling 4- to 5-month-old female rats were mated with fertile males and the day on which vaginal smears showed sperms was considered day 1 of pregnancy. Laparotomy was performed on rats on day 7 of pregnancy and the total number of implantation sites in the uterine horns counted. Starting from day 10, for 3 consecutive days, 3 mg of affinity-purified IgG/animal/day was administered through the intraperitoneal route. At the end of gestation period (21 days), the number of pups delivered were recorded. For active immunization, 3-month-old fertile female rats were primed with 200 μg of the individual peptides emulsified in FCA through subcutaneous route for primary injection. For subsequent boosters, 100 μg of corresponding peptide emulsified in FIA was given at 3-week intervals. After the third booster, test bleeds were collected through cardiac puncture. These sera were analyzed for immunoreactivity with the respective peptide as well as the native RCP by ELISA. The animals were then mated with proven fertile male rats. The day on which sperms were detected in the vaginal smears was taken as day 1 of pregnancy. Another booster dose with the corresponding peptide was given on day 1 and pregnancies were monitored.

RESULTS

Binding Characteristics of Synthetic Peptides to mAb 6B2C12

The measurement of the antigenic characteristics of the parent C-20 as well as the engineered HE-20 pep-

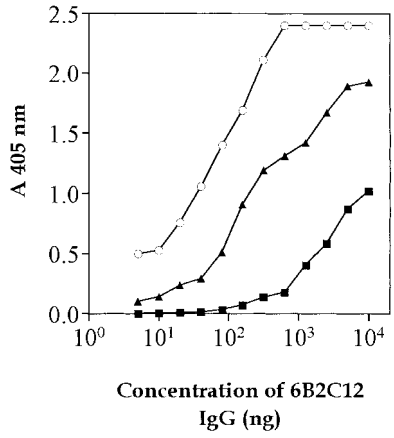

FIG. 2. Reactivity of the mAb 6B2C12 as determined by ELISA. Shown are the antibody dilution curves obtained with 2 μg/well of C-20 (■), HE-20 (▲), or 1 μg/well of native RCP (○). The antigen–antibody interaction was visualized by employing alkaline phosphatase conjugated goat-anti-mouse IgG and *p*-nitrophenyl phosphate as the chromogen. Color development was monitored at 405 nm.

tide was carried out by ELISA using the mAb 6B2C12 raised against native RCP (8). Antigen binding patterns as a function of antibody dilution (Fig. 2) clearly show that, in addition to native RCP, both the peptides are recognized by this mAb in a dose-dependent manner. As anticipated, the native RCP was the most efficient in this respect requiring approximately 50 ng for 1/2 B_{max} binding. This is followed by the engineered peptide HE-20 (220 ng), C-20 being least efficient (1900 ng). This order of binding presumably represents the propensity to assume ordered conformation recognized by the paratope on the mAb 6B2C12. It may be relevant to mention that the helical content of HE-20 calculated from CD spectral measurements in 50% aqueous TFE and expressed as the molar ellipticity θ_M in deg.cm^2 day mol^{-1} at 222 nm was higher at −65,000 vis-a-vis C-20 (−10,000) (10).

Mapping of T-Cell Epitope in C-20

The recent demonstration that the parent C-20 peptide of chicken RCP per se can function as an epitope-based vaccine i.e., without carrier conjugation to elicit anti-peptide antibodies recognizing native RCP implied that it harbors a functional T-cell epitope within its 20-amino-acid sequence of ^{200}HACQKKLLKFEALQQEEGEE^{219} (20). It was therefore considered important to map the T-cell epitope in C-20 to find whether any significant change in the nature of T-cell determinant has occurred during peptide engineering. To map the boundaries of the T-cell epitope, truncated peptides were synthesized with deletion of amino acids from the N-terminal end of C-20 peptide. These include a 15-mer (KLLKFEALQQEEGEE) and a 12-mer (KFEALQQEEGEE). When these peptides were tested for T cell proliferation *in vitro* (Fig. 3), it was clear that both 20-mer and 15-mer could effectively stimulate the proliferation of T-lymphocytes in culture from C-20 primed animals whereas the 12-mer peptide was ineffective. This indicates that the critical region for interaction with T-cell receptor is centered around the sequence KLL and deletion of the N-terminal 5 residues (HACQK) did not adversely affect the proliferation thereby demonstrating redundancy of these residues for T-cell receptor interaction. On the other hand, such a deletion enhanced cell proliferation by 1.5-fold vis-a-vis the parent peptide. While the mechanism underlying enhanced proliferation with the truncated peptide awaits further study, this finding is reminiscent of the observation with the T-cell antigenic determinant of Staphylococcus nuclease (residues 91–105) wherein deletion of 5 residues at C terminus resulted in greater T cell stimulatory activity *in vitro* (21). It is noteworthy that the T-cell epitope mapped, in C-20 overlaps with the core sequence (QKKLLKFEAL) of the B-cell epitope and both these epitopic sequences remain conserved during peptide engineering to enhance helix stabilization in HE-20. It was therefore anticipated that HE-20 would also function as a minivaccine to elicit peptide antibodies cross reactive with RCP.

To confirm this and to assess the relative efficacy of the two peptide analogues, *in vitro* lymphocyte proliferation assays were carried out. The results depicted in Fig. 4 clearly show that HE indeed harbors a T cell epitope with apparently higher recall response compared to C-20. The reason for higher proliferation index achieved with HE-20 awaits further study and may be attributable to modifications introduced in sequences flanking the T cell epitope during peptide design to stabilize helicity. In parallel experiments we have observed (data not shown) that another engi-

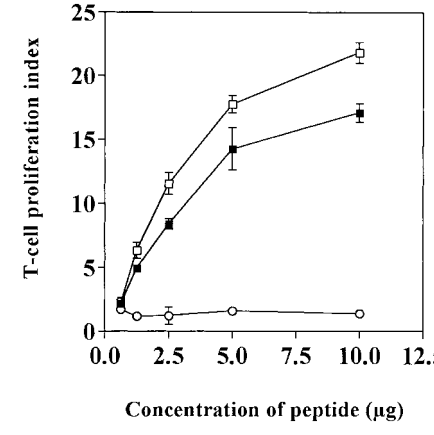

FIG. 3. Proliferation of T-lymphocytes from rats immunized with C-20 peptide. Adult rats were immunized with 100 μg of the peptide emulsified in FCA in the hind foot pads. After 10 days, popliteal lymph node cells were collected and incubated with serial dilutions of C-20 (■), 15-mer (□), or 12-mer (○). T-cell proliferation indices were determined after 4 days of culture. Values represent means ± SD of triplicate cultures from three different experiments.

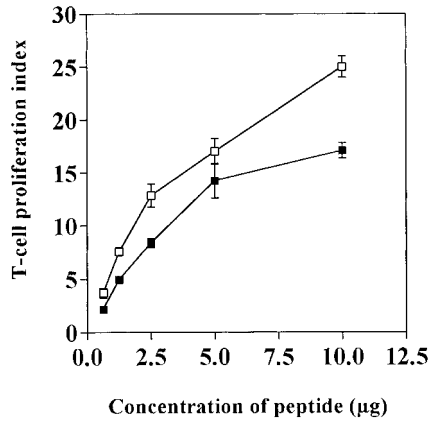

FIG. 4. Proliferation of T-lymphocytes from rats immunized with C-20 (■) or HE-20 (□) peptide. Popliteal lymph node cells collected from immunized animals were incubated with serial dilutions of the respective antigens. T-cell proliferation indices were determined after 4 days in culture. Values represent means ± SD of triplicate cultures from three independent experiments.

neered peptide HC-20, with substitutions restricted to C terminus to B and T cell epitope is equipotent with C-20 in eliciting T cell proliferation. This implies that replacement of Cys with Glu in HE-20 is responsible for the enhanced proliferation index and accords with the higher response observed with truncated 15 mer peptide lacking cystine residue. Of relevance in this context is the observation that substitutions of the cysteine residues of hen egg white lysozyme peptide (residues 74–88) with serine enhances T-cell stimulation of the I-A^b restricted T-cell hybridomas without significant alterations in the MHC class II binding (22).

Relative Immunogenicities of C-20 and HE-20

Having established the presence of a common T-cell epitope in the two analogs, we chose to use these free peptides as immunogens to assess their relative efficacies in terms of eliciting anti-peptide and anti-RCP antibodies in rabbits in terms of titers and affinities. The dilutions of the antisera required for 50% of the binding maxima against the individual peptides and the native RCP as measure of titers along with their affinity constants (determined from the molar concentrations required for 50% inhibition of binding in competitive ELISA) are presented in Table 1. Rabbit anti-peptide sera, besides specifically and preferentially recognizing the respective parent peptides, also cross-reacted significantly with the other peptide as well as with RCP (Fig. 5). From the patterns of displacement curves so obtained, it is clear that the two anti-peptide antibodies exhibit significant differences in terms of reactivity with their cognate antigen and this extends to their binding to native RCP. It is also obvious that anti-HE-20 antibodies harbor a higher proportion of RCP crossreactive antibodies vis-a-vis anti-C-20 antibodies. Furthermore the affinity of anti-HE-20 antibodies to bind native protein was 100-fold higher than the anti-C-20 antibodies, in conformity with earlier results obtained with DT-conjugated peptides (15). Thus, the superiority of HE-20 over C-20 to elicit immune response to generate specific antibodies cross-reactive with the native RCP manifests both qualitatively (affinity) and quantitatively (titer) commensurate with its relative structural stability induced due to peptide engineering.

TABLE 1

Characteristics of Rabbit Antipeptide Sera

	Antiserum titers[a] against		Affinity constant (M^{-1})	
Immunogen	Immunogen	RCP	Immunogen	RCP
C-20	1:48,000	1:26,000	3.6×10^9	0.9×10^8
HE-20	1:72,000	1:40,000	4.3×10^{10}	1.1×10^{10}

[a] The titer is defined as the dilution of antiserum giving a value of approximately 1.5 OD units at 405 nm against peptide or RCP in an ELISA.

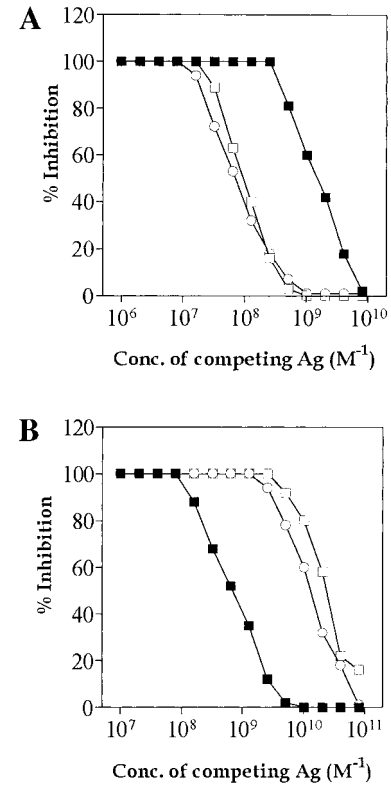

FIG. 5. Inhibition of binding of rabbit anti-peptide sera to the individual peptides and chicken RCP. Inhibition curves obtained with rabbit anti-C-20 serum (A) and rabbit anti-HE-20 serum (B) are shown. Varying concentrations of C-20 (■), HE-20 (□), or chicken RCP (○) were incubated with polyclonal antiserum for 2 h at 37°C. They were then added to the corresponding peptide immobilized wells. The extent of inhibition in percentage was plotted on *Y*-axis.

TABLE 2

Effect of Passive Immunoneutralization of Endogenous RCP with Antipeptide IgG on Pregnancy Progression in Rats

Treatment[a] (n = 4)	Number of implantation sites on day 7 (NT/T)	Remarks (NP)
Preimmune IgG	40/10	Delivered pups (36)
Anti-C-20 IgG	33/8	No pups (0)
Anti-HE-20 IgG	36/9	No pups (0)

Note. NT, total number of implantation sites; T, average number of implantation sites/animal as determined by laparotomy; NP, total number of pups born, given in parentheses.

[a] The pregnant rats were treated with either preimmune IgG or antipeptide IgG (3 mg/day/animal, ip) for 3 consecutive days on day 10, 11, and 12 of pregnancy.

Bioefficacy of Anti-peptide Antibodies Following Passive Immunization in Pregnant Rats

The effect of these anti-peptide antibodies on pregnancy progression was evaluated by administering the protein A–Sepharose affinity purified individual IgG to proven pregnant rats on days 10, 11, and 12. Normal rabbit IgG were administered to control animals. Pregnancy progression was monitored and the number of pups born at term is given in Table 2. It is of significance that antibodies to the modified analog HE-20 is equally effective in precipitating embryonic resorption in the rats and hence early interruption of pregnancy establishment.

Active Immunization with the Peptide Analogs and Effect on the Magnitude and Duration of Contraceptive Efficacy

To confirm and compare the neutralizing propensities of the antibodies elicited by HE-20 vis-a-vis C-20 as minivaccines, active immunization studies were carried out in fertile female rats. Toward this, 3-month-old female rats were immunized with the peptides individually at three weekly intervals. Ten days after the third booster, the sera were analyzed for specific antibodies. The titers of these rat anti-peptide sera against the individual free peptide as well as with the native RCP were estimated by direct ELISA (Table 3). It is very clear from these results that both the peptide analogs are highly immunogenic in the rat as in the rabbit and produce anti-peptide antibodies, significant populations of which also recognize the native RCP. When the circulatory antibodies reached their plateau, these immunized female rats were mated with proven fertile, age-matched males. The day on which sperms were detected in the vaginal smears was taken as day 1 of pregnancy. A booster dose of the corresponding peptide was given on that day and the pregnancy progression was monitored till term. Initially, 100% curtailment of pregnancy was observed in the rats immunized with either HE-20 or C-20 (Table 3). Interestingly, when the animals were put through second round of mating, it was observed that HE-20 immunized animals continued to harbor significant levels of anti-peptide and anti-RCP antibodies without further boosting even after 6 months following the last injection. In contrast, during this interval animals from the C-20 immunized group harboring low or nonmeasurable antibody titers delivered healthy off-springs, indicative of reversibility of the contraceptive effect. These observations prove that the engineered peptide analogue with greater propensity to assume helical conformation elicits longer lasting and higher affinity antibodies in circulation which manifests in prolonged contraceptive efficacy. Similar experiments with BALB/c mice have essentially confirmed the above observations (data not shown).

DISCUSSION

It is now recognized that B-cell response to peptide antigens is governed by multiple parameters such as T-cell dependency, epitopic structure and stability, adjuvanticity etc. The ability of short synthetic peptides to induce antibodies which bind the native protein with reasonable affinity has been rationalized on the basis of the propensity of such antigenic determinants to adopt appropriate conformations resembling those ex-

TABLE 3

Influence of Active Immunization with C-21 and Its Analog HE-21 on Fertility Performance in Female Rats

	Antiserum titers against (10 days after third booster)			Antiserum titers against (180 days after third booster)		
Immunogen	Immunogen	RCP	Result (NP)	Immunogen	RCP	Result (NP)
C-20	1:8400 ± 1200	1:4800 ± 620	Did not deliver (0)	1:200 ± 100	ND	Delivered pups (48)
HE-20	1:12800 ± 1600	1:8000 ± 1400	Did not deliver (0)	1:6000 ± 800	1:3200 ± 600	Did not deliver (0)

Note. Values are means ± SD of the sera collected from six rats from each group. NP, total number of pups born, given in parentheses; ND, not detectable.

hibited where it is covalently linked in the protein, either in solution or when bound to B-cell surface receptor (23). In fact, a significant correlation has been observed between peptide immunogenicity and the tendency to adopt folded backbone conformations characteristic of the corresponding site on the protein despite the fact that short linear peptidyl sequences generally lack well-ordered structure in aqueous solution (24–29). In the present study, we have examined whether there exists any direct relationship between structure and immunogenic/antigenic characteristics of C-20 of the avian riboflavin carrier protein vis-a-vis its homolog HE-20. This peptide was engineered to enhance the helical content such that core sequences corresponding to both B- and T-cell epitopes are unaffected.

Our previous CD spectroscopic studies have shown that C-20 exhibits measurable α-helical propensity in helix-inducing solvents like 20% aqueous TFE (15) in conformity with our earlier algorithm-based theoretical prediction (30). This has been recently confirmed by X-ray crystal structure analysis of the native RCP at 2.8 Å resolution which identified the segment of residues 202–210 as α helical (33). As stated earlier, our approach to enhance helix stability in C-20 involved stepwise introduction of $i \rightarrow i + 3/i + 4$ salt bridges in the flanking sequences of the core B-cell epitopic sequence ^{203}QKKLLKFEAL^{212} which resulted in progressive increase in affinities of the anti-peptide antibodies to RCP. However, such anti-peptide antibodies were generated in animals immunized with the peptide conjugated to DT as carrier protein. One potential pitfall in such studies is the likely influence of the carrier per se on peptide conformation which may vary with the local environment surrounding different sites of peptide conjugation; additional factors that govern peptide immunogenicity consequent on carrier conjugation following repeated boosting have been recognized (3, 33). Our recent demonstration that C-20 can be folded as an amphipathic helix and can elicit anti-peptide and anti-RCP antibodies with significant contraceptive potential in rodents to function as minivaccine (20) prompted characterization of the minimal or "core" T-cell epitopic sequence harbored by C-20 as well as HE-20. Using lymph node cells primed with C-20 *in vivo* and truncated peptides for recall stimulation of T-cell proliferation, it was intriguing to find that the five amino acids (HACQK) at the N-terminus are redundant for *in vitro* stimulation. This has further been substantiated by similar experiments employing a series of 15-mer peptides (starting from N-terminus and overlapping by one amino acid each) which identified the core sequence of T-cell epitope as "KLLKFEALQQ" (unpublished observations) overlapping with core B-cell epitope "QKKLLKFEAL." Relatively higher T-cell proliferation observed *in vitro* with the truncated peptides may reflect either a requirement for C-20 processing through endocytotic route for optimal epitope presentation in an MHC-restricted manner on APC surface or steric hindrance contributed by the flanking sequence N-terminal to the core epitope (34). The presence of cys residue in this region, susceptible to cysteinylation thus necessitating exposure to reducing endosomal environment prior to presentation at the APC cell surface (35) may be a contributing factor in this regard. Higher proliferation observed with HE-20 in which Cys residue replaced with Glu vis-a-vis C-20 then is to be anticipated since the T-cell epitope remains unaltered in its structural design. This might account for its more efficient MHC-II-restricted functional display on APC for efficient T cell receptor activated T-cell proliferation. Additional investigations involving aldehyde-fixed enriched B cells as APC should clarify the underlying mechanism.

Our observation that helical scaffolding of C-20 sequence results in relatively higher binding of HE-20 to the paratope of RCP-specific mAb 6B2C12 (Fig. 2) correlates with its improved potential to mimic the native structural motif at this site in RCP, presumably due to lesser free energy required for interaction through induced fit. This antigenic superiority of HE-20 might have also contributed significantly to its enhanced immunogenicity (Fig. 5). It is now established that during maturation of immune response, progression of antigen-activated B-cell function through various stages is driven by cognate interaction with antigen specific T-cells and is critical for sustained humoral response (36). During this process, B-cells bind antigens through their surface IgG receptor as high affinity trapping sites for eventual epitope presentation to T-cells in MHC-II restricted mode, and function as the most efficient APC particularly under limiting antigen supply. Hence, the stability and affinity of their surface IgGs for antigenic epitopes, which in turn influence ligand density at APC surface, would eventually determine the quality and quantum of T-cell help (37). Our data (Fig. 2) demonstrating higher HE-20 binding to mAb 6B2C12 may thus be a direct reflection of increased ability of HE-20 polyclonal antibody to bind RCP, since surface IgG on B-cell and that secreted by the plasma cells are products of the same affinity maturation process. Thus the lower immune response of C-20 in terms of binding to RCP can then be rationalized in structural terms i.e., its lesser propensity to mimic the corresponding native structure on RCP compared to HE-20. Additional contributory factors for higher immunogenicity of HE-20 may include relative resistance to proteolytic degradation and the enhanced ability of amphipathic peptides presented in helical fold to intercalate into membranes (38). The increased affinity as well as the population of the antibodies that recognize native RCP following HE-20 immunization would then account for prolonged contraceptive effect observed in the rodent model.

In summary, the above studies have demonstrated that it is possible to design a peptide antigen with predetermined conformational propensity, structurally characterize its properties, elicit higher affinity and titer antibodies without coupling to carrier proteins to generate protein-reactive antibodies by secondary structure stabilization using rational design principles such that modifications introduced do not disturb its inherent immunological potential. In the past, several attempts to engineer antigenic determinants have mainly focused on supersecondary structures often employing designed sequences of 35–80 residues long (11–14) while the present strategy employed secondary structure stabilization of a relatively short fragment of 20 amino acids adopting simple design principles. Efforts are currently underway to extend these studies to subhuman primate model to explore the applicability of the engineered peptide as a contraceptive minivaccine in primates.

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