

Immunocontraceptive potential of recombinantly expressed minimized chicken riboflavin carrier protein (mini-RCP) in rodents

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Chicken riboflavin carrier protein (RCP; 219 AA) harbours four linear epitopes, constituted by the peptide residues 3-23, 64-83, 130-147 and 200-219. Antibodies to these sequences bionutralize maternal RCP and provide protection from pregnancy in rodents. In order to overcome the major histocompatibility complex-dependent variability in immune response often encountered with use of single peptides for vaccination in genetically outbred populations, we have assembled a novel synthetic gene, incorporating in tandem the nucleotide sequences coding for all the four neutralizing epitopes of chicken RCP and expressed in *Escherichia coli*. The gene product, mini-RCP has been characterized for its immunogenic properties and contraceptive potential in rodents. Immunization of rabbits and rats led to generation of antibodies against individual peptide components, as determined by enzyme-linked-immunosorbent assay (ELISA). However, immunized rats carried pregnancy to term and delivered healthy offsprings. Antisera from these rats exhibited decreased affinity of binding to the native protein. These findings suggest that the prospects of covalently-linked epitope peptides need to be cautiously evaluated during the design and development of peptide-based vaccines.

Keywords: Immunocontraception, immunogenicity, chicken riboflavin carrier protein, major histocompatibility complex, minimized riboflavin carrier protein, synthetic gene, rat, rabbit

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Chicken riboflavin carrier protein (RCP) is an effective immuno-contraceptive vaccine in rodents and sub-human primates¹. However, not all the sub-populations of the antibodies are relevant in the context of immuno-interception of pregnancy. Primary amino acid (219 AA) sequence of chicken RCP harbours six major linear B-cell epitopes spanning the residues 10-17, 42-49, 68-83, 134-141, 172-179 and 200-207, immuno-dominant in mice, rats, rabbits and monkeys². Larger synthetic oligopeptides (18-21-mers) harbouring these 'core' B-cell epitopes with N- or C-terminal extensions function as mini-vaccines in terms of eliciting peptide-specific, protein-cross-reactive antibodies^{3,4}. Among these, four peptides (residues 3-23, 64-83,

130-147 and 200-219, abbreviated as YGC, CED, GEN and HAC, respectively) elicit neutralizing antibodies capable of curtailing pregnancy in rodents. However, antibodies directed to residues 33-49 (CYA peptide) or 169-186 (CLQ peptide), despite being immuno-dominant do not interfere with pregnancy in rodents^{3,4}.

In the design and development of vaccines and antisera, the most widely used strategy involves preparation of antisera against synthetic peptide immunogens. However, use of individual peptides for vaccination may not always be appropriate, since certain members of a population may not respond due to major histocompatibility complex (MHC)-restriction. Thus, it may be necessary to develop several peptides for each vaccine. Towards this end, we designed earlier, a novel synthetic gene (mini-RCP), incorporating in tandem the nucleotide sequences coding for all the four neutralizing epitopes of chicken RCP⁵, which has a potential to encode an 86 AA peptide (Fig. 1). The N-terminal 2-22 residues encoded YGC peptide. The residues 25-44, 47-64, and 67-86 corresponding to the amino acid sequences of peptides CED, GEN and HAC, respectively arranged in the

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Abbreviations used: RCP, riboflavin carrier protein; MHC, major histocompatibility complex; PCR, polymerase chain reaction; IPTG, isopropyl thiogalactoside; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline.

M¹YGCLEGDTH¹⁰KANPSPEPNM²⁰
 HELACEDFTK³⁰KIEAFYRASP⁴⁰
 HAARLAGENH⁵⁰CKSKAVPYSE⁶⁰
 MYANLAHACQ⁷⁰KLLKFEALQ⁸⁰
 QEEGEE⁸⁶

Fig. 1—Primary amino acid sequence of recombinantly expressed mini-RCP represented in single letter code

order they are located in the native RCP⁵. A leucyl alanine linker (AA 23-24, 45-46 and 65-66) was introduced between these peptidyl regions with a view to facilitate the limited cleavage by intracellular proteases, such as cathepsins during antigen processing⁶ through endosomal/lysosomal pathway prior to display of the resulting fragments on their surface in an MHC-restricted mode. In the present communication, the presence of functional T-cell epitopes present in the individual peptide sequences was established. In addition, the influence of mini-RCP antibodies on pregnancy establishment was evaluated.

Materials and Methods

Animals

Wistar rats and albino rabbits procured from the Central Animal Facility at Indian Institute of Science, Bangalore were fed pelleted diet and water *ad libitum*. They were exposed to 12 hr light/12 hr dark schedule under controlled conditions of humidity and ambient temperature.

Materials

Oligonucleotides were custom synthesized by Microsynth (Balgach, Switzerland). All the synthetic peptides were custom synthesized by Mimotopes Pty Ltd. (Australia) and their individual purities (>95%) were established by reverse-phase HPLC. Restriction enzymes were purchased from MBI Fermentas (Vilnius, Lithuania). Plasmid pRSET A was procured from Invitrogen (Carlsbad, CA, USA). Isopropyl thiogalactoside (IPTG), Ni CAM HC resin, imidazole, goat anti-rabbit and anti-rat IgG conjugated to alkaline phosphatase, bovine serum albumin (fraction V), *p*-nitrophenyl phosphate, Freund's complete and incomplete adjuvants (FCA/FIA) were from Sigma Chemical Co., St. Louis, USA. Protein A-Sepharose was obtained from Pharmacia Fine Chemicals (Sweden) and [³H]-thymidine from Bhabha Atomic Research Centre, Mumbai. Phytohaemagglutinin, fetal bovine serum and RPMI 1640 were procured from GIBCO laboratories (USA). High-binding microtitre

plates were purchased from Greiner Labortechnik Ltd. (Germany). Chicken RCP was purified to homogeneity from chicken egg white⁷. Promega wizard mini columns for PCR amplified DNA purification were from Promega Biotech (Madison, WI, USA).

Synthesis, cloning and expression of mini-RCP

The approach adopted in the synthesis of mini-RCP gene was similar to that described earlier⁸. The gene encoding mini-RCP was engineered by contiguous alignment of nucleotide sequences coding for all the four neutralizing epitopes of chicken RCP separated by leucyl alanine residues. Synthesis, cloning and expression of mini-RCP gene was performed as per described protocols⁵. Briefly, the nucleotide sequence has been divided into six oligonucleotides, ranging in length from 38 to 60 bases for a total of 264 bases. Two end primers of 30-nucleotide length were employed to introduce the recognition sites for Xho I and EcoR I at 5' and 3' ends, respectively. Overlapping PCR were performed for 20 cycles. Each cycle consisted of a 1 min denaturation at 96°C, followed by 1 min annealing (at 50°C for the synthesis of "core template" and at 45, 48 and 56°C for the II, III and IV rounds of PCR, respectively) and by a 2 min extension at 72°C. The final PCR product was purified through Promega wizard minicolumns as per manufacturer's instructions. Bacterial cultures, plasmid purification, and transformations were performed using standard protocols⁵. An aliquot of the purified DNA was subcloned into pRSET A vector. Expression of mini-RCP was achieved by transformation and induction of BL 21 (DE3) pLys S cells with 1 mM IPTG. The recombinant protein was purified to homogeneity by a single step Ni²⁺ affinity chromatography. Homogeneity of the purified protein was established by SDS-PAGE and N-terminal amino acid sequencing.

T-cell proliferation assay

Lymphocyte stimulation assays were performed as described⁹. Briefly, 3-months-old rats were injected with 100 µg of mini-RCP emulsified in FCA, subcutaneously, in the hind foot pads. After 15 days, popliteal lymph nodes were aseptically dissected and single cell suspensions prepared. About 4 × 10⁵ 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.

Phytohaemagglutinin (5 μ l/well) was used as a positive control. The assay was performed in triplicate in 96-well microtitre plates. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. After an 18 hr pulse with 1 μ Ci [³H]-thymidine per well at the end of a 4-day incubation, 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 were presented as T-cell proliferative indices, calculated as the [³H]-thymidine incorporation by cells cultured with the peptide relative to the [³H]-thymidine incorporation by cells cultured in the absence of the peptide.

Production of polyclonal antibodies

Mini-RCP emulsified in FCA was injected into rabbits at a dose of 500 μ g/animal for primary injection. For subsequent boosters at 3-week intervals, 250 μ g of protein emulsified in FIA was administered subcutaneously. After each booster, the rabbits were bled, sera collected and stored in aliquots at -20°C until use.

Characterization of the antisera

The immune recognition patterns of these antisera towards mini-protein, the individual free constituent peptides as well as the native RCP were determined by direct ELISA¹⁰. Briefly, either 2 μ g of peptide or 1 μ g of protein were coated in a high binding ELISA plate. After blocking the unoccupied sites with 0.3% (w/v) BSA, these wells were probed with serially diluted antiserum. The antigen-antibody complex was visualized by employing alkaline phosphatase conjugated goat anti-rabbit IgG or anti-rat IgG and *p*-nitrophenyl phosphate as chromogen. The colour developed was monitored at 405 nm. The affinity of these antisera to mini-RCP, individual peptides and native RCP was determined by inhibition ELISA¹¹.

Purification of IgG

Mini-RCP antiserum was dialyzed against 0.1 M phosphate, pH 8.0 and loaded on to protein A-Sepharose affinity column pre-equilibrated with the same buffer. The bound IgG was eluted with 0.1 M glycine, pH 3.0. Fractions of 1 ml were collected into tubes containing 20 μ l of 2 M Tris (to immediately neutralize the pH) and fractions showing absorbance at 280 nm were pooled, dialyzed against PBS and checked for binding to the protein by ELISA.

Immunoneutralization studies

For passive immunization, regularly cycling 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 three consecutive days, 3 mg of affinity-purified IgG/animal/day was administered through intraperitoneal route. At the end of gestation (21 days), the number of pups delivered was recorded.

For active immunization studies, 3-months old fertile female rats were recruited and sensitized with 200 μ g of mini-RCP emulsified in FCA through the sub-cutaneous route for primary injection. For subsequent boosters, 100 μ g of mini-protein emulsified in FIA was given at 3-weeks intervals. After the third booster, test bleeds were collected through cardiac puncture. The sera were analyzed for immunoreactivity with mini-protein, individual peptides and native protein by ELISA. Animals were then mated with proven fertile male rats and the day on which sperms were detected in the vaginal smears was taken as day 1 of pregnancy. Another booster dose with the mini-protein was given on day 1 and the successful pregnancies were monitored at term by counting litters.

Results and Discussion

The choice of the mini-protein, instead of the whole protein of chicken RCP was primarily based on our earlier observations^{3,4} that at least two other regions elicit high affinity and titre antibodies and yet fail to interfere with progression of pregnancy in rodents. We also demonstrated earlier that each of the four peptide sequences included in the mini-RCP harbours a functional T-cell epitope along with an overlapping/proximal B-cell recognition site within them^{3,4}. The presence in tandem of all the four neutralizing epitopes along with respective overlapping T-cell determinants in this multi-determinant mini-RCP might aid in overcoming the limitations often encountered with single peptide-based vaccinations in genetically diverse populations.

The substrate specificity of cathepsin D from various sources indicates that it preferably cleaves peptide bonds, linking two hydrophobic amino acid residues⁶. These findings suggest an important role for cathepsin D in the processing of protein antigens, an essential step for their recognition by T-cells. It is, therefore, anticipated that the inclusion of leucyl alanine spacers between the peptidyl sequences would focus proteolysis during antigen processing to these

sites without curtailing generation of the previously characterized T-cell epitopes.

Proliferative T-cell responses against mini-RCP

Popliteal lymph node cells collected from mini-RCP immunized rats were *in vitro* re-stimulated with individual constituent peptides and the proliferative indices achieved are shown in Fig. 2. Immune lymph node cells proliferated well in a dose-dependent manner in the presence of any one of the peptides, mini-RCP or native RCP. However, responses to the mini-protein were higher, as compared to the proliferative responses induced by single peptides, which may be attributed to generation of junctional epitopes created after the covalent linkage of the constituent peptides. In certain instances¹²⁻¹⁴, the covalent linkage of two T-cell epitopes resulted in intramolecular competition between the two epitopes for MHC binding, thus directing the immune response predominantly towards the immuno-dominant T-cell epitope. However, in the present study, all the four individual constituent peptides brought about induction of T-cell proliferation to a similar extent, indicating the absence of intramolecular competition among them.

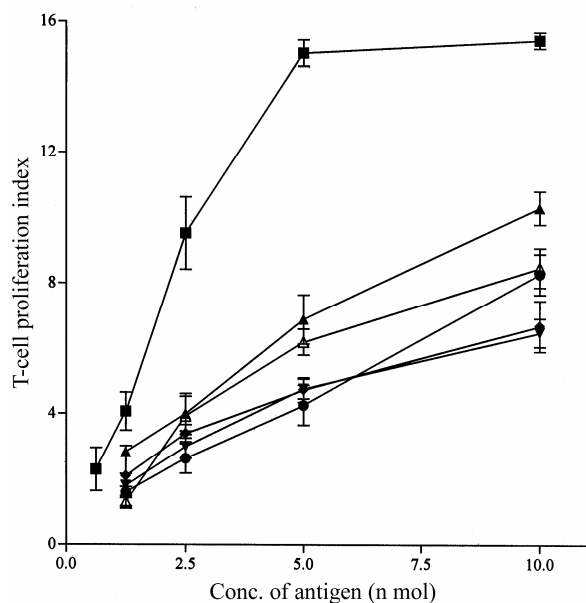


Fig. 2—Proliferation of T-lymphocytes from rats immunized with mini-RCP [Adult rats were immunized with 10 n mol of the antigen emulsified in FCA in the hind foot pads. After 15 days, the popliteal lymph node cells were collected and incubated with serial dilutions of either YGC (▲), CED (▼), GEN (◆), HAC (●), mini-RCP (■) or native RCP (△). T-cell proliferation indices were determined after 4 days of culture. Values represent the mean \pm S. D. of triplicate cultures from 3 different experiments]

Characterization of anti-mini-RCP antibodies

Having thus ascertained that mini-RCP indeed harbours functional T-cell recognition sites, female rats and rabbits were immunized with mini-RCP to elicit antibody production in both the species. The ability of rabbit anti-mini-RCP sera to recognize the constitutive peptides as well as the native RCP was assessed by ELISA (Fig. 3). It is evident from the binding curves that these polyclonal antibodies, in addition to reacting with the homologous antigen, bound to their cognate epitopes on the individual free peptides as well as the native protein, in a dose-dependent manner. The parallelism between the curves shows that they share similar epitopic conformations on their surface. The results indicate that mini-RCP was two-fold more efficient than the native protein, in terms of the dilution of antiserum required for 50% Bmax.

Influence of passive immunization with anti-mini-RCP antibodies on pregnant rats

In order to assess the influence of these antibodies on pregnancy progression, the protein A-Sepharose purified IgG were administered to pregnant rats on three consecutive days (i.e., on day 10, 11 and 12). The animals receiving non-immune IgG at equivalent doses served as controls. The effects on pregnancy progression were monitored at term and the numbers of pups born are presented in Table 1. It is very intriguing to find that anti-mini-RCP IgG did not exhibit any effect on progression of pregnancy, since all the six animals delivered healthy offsprings with litter size comparable to those in control animals.

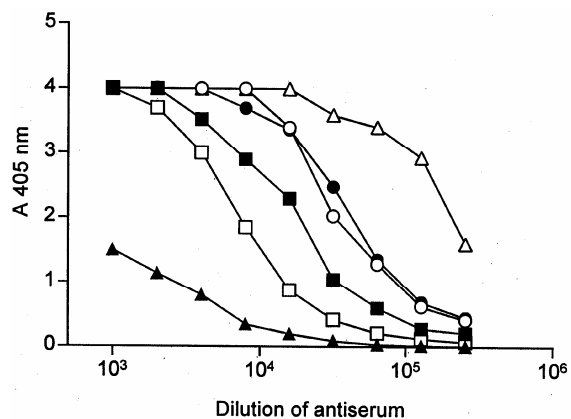


Fig. 3—Reactivity of rabbit polyclonal anti-mini-RCP sera as determined by ELISA [Antibody dilution curves obtained with the peptide YGC (■), CED (▲), GEN (□), HAC (○), mini-RCP (▲) and native RCP (●) are shown]

Table 1—Effect of passive immunoneutralization of endogenous RCP with anti-mini-RCP IgG on pregnancy progression in rats
[The pregnant rats were treated with IgG (3 mg/day/animal, i.p.) for 3 consecutive days on day 10, 11 and 12 of pregnancy, n=6]

Treatment	Number of implantation sites on day 7 (nT/T)	Remarks (nP)
Pre-immune IgG	54/9	Delivered pups (46)
Anti-mini-RCP IgG	49/8	Delivered pups (44)

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

Table 2—Influence of active immunization with mini-RCP on fertility performance in rats and characterization of rat anti-mini-RCP sera

Rat no.	Antiserum titre* against						Result (nP)	Affinity constant (M^{-1})	
	YGC	CED	GEN	HAC	RCP	mini-RCP		mini-RCP	RCP
1	1:2000	1:4000	1:3000	1:1000	1:16000	1:25000	Delivered (8)	9.6×10^8	3.6×10^6
2	1:2000	1:3200	1:3000	1:8000	1:32000	1:50000	Delivered (12)	8.4×10^8	4.2×10^6
3	1:2000	1:2000	1:3000	1:3000	1:8000	1:12800	Delivered (9)	1.5×10^9	8.7×10^5
4	1:4000	1:8000	1:1000	1:1200	1:20000	1:40000	Delivered (7)	7.2×10^8	9.6×10^5
5	1:4000	1:2000	1:1600	1:1600	1:32000	1:50000	Delivered (6)	1.0×10^9	6.7×10^6
6	1:8000	1:5000	1:1000	1:1000	1:40000	1:60000	Delivered (11)	1.2×10^{10}	5.4×10^6

* The titre is defined as the dilution of antiserum giving a value of approx. 1.0 OD unit at 405 nm against peptide or RCP in an ELISA

nP, Total number of pups born, given in parentheses

Effect of active immunization with mini-RCP on pregnancy establishment in rats

The inability of minimized protein to bionutralize maternal RCP was further confirmed by active immunization studies. Towards this, 3-months-old female rats were immunized with mini-RCP. The immune sera were collected from these animals on the tenth day after the third booster and characterized by ELISA. The titres of these antibodies against the free peptides *vis-a-vis* the native protein are presented in Table 2. It is very clear from these results that the truncated RCP is highly immunogenic in rats and rabbits and produce antibodies, a significant population of which also recognizes the constituent peptides and native protein. When circulatory antibodies were sufficiently high, these immunized female rats were mated with proven fertile, age-matched male rats. The day on which sperms were detected in the vaginal smears was taken as day 1 of pregnancy. A booster dose with mini-RCP was given on that day and the pregnancy progression was observed. The results (Table 2) demonstrate that mini-RCP failed to confer protection from pregnancy, despite harbouring the sequences corresponding to four bionutralizing linear epitopes of chicken RCP. These observations corroborate the outcome from experiments employing passive immunization approach. Since affinity for the antigen is one of the prime parameters of the antibody response that

markedly affects the biological activity¹¹, we measured the affinity of mini-RCP antibodies to individual peptides and native RCP. The results (Table 2) support the possibility of relatively lower affinity binding as one of the causes underlying the failure of mini-RCP antibodies to interfere with pregnancy progression.

Proliferative T-cell responses with truncated peptides

While designing a peptide assembly-based vaccine, it is necessary to ensure that the recombinant antigen mimics the structure in its native form. In addition, the processing of T-cell epitopes can be affected by antigen conformation or amino acid substitutions in flanking regions or at distant sites in the primary structure^{15,16}. It was observed (Fig. 2) that the response of T-cells was generally greater when stimulated by the mini-RCP than when stimulated either by the individual peptides or the full length native protein. This could be attributed to generation of junctional/additional epitopes¹² or to enhancement of the response by the new flanking residues in the joined peptides¹⁷. In either case, the response directed towards individual parent T-cell epitopes was not inhibited.

In order to study the effect of the linear assembly of the earlier defined T-cell epitopes, lymphocyte proliferation assays were carried out with truncated

Sequence	Nomenclature
YGCLEGDTHKANPSP	ycg
YGCLEGDTHKANPSPEPNMHE	YGC
DFTKKIEAFYRASPH	ced
CEDFTKKIEAFYRASPHAAR	CED
HCKSKAVPYSEMYAN	gen
GENHCKSKAVPYSEMYAN	GEN
QKLLKFEALQEEG	hac
HACQKLLKFEALQEEGEE	HAC

Fig. 4—Primary amino acid sequence of the peptides used in the study [The corresponding truncated peptides are abbreviated in lower case letters]

peptides (Fig. 4), corresponding to each individual full-length peptide. With the exception of the truncated overlapping peptides corresponding to the sequence of YGC, all the remaining shorter peptides of CED, GEN and HAC sequences failed to induce lymphocyte proliferation *in vitro* (Fig. 5). However, peptides corresponding to ygc sequence stimulated proliferation of lymphocytes *albeit* to a lesser extent when compared to parent YGC peptide. It is pertinent to mention that these truncated 15-mer peptides induced effective T-cell proliferation when the animals were immunized either with individual parent peptides or native RCP¹⁸. Hence, it appears that the stimulation observed with parent peptides is due to the presence of a partial T-cell epitope within their sequence complete epitopic sequence of which has been newly generated in the linearly assembled mini-RCP. A similar diversion of the proliferative T-cell responses was observed after immunization with a chimeric peptide composed of an immunodominant Ac1-11 mouse myelin basic protein epitope, attached to the 35-47 epitope from the same molecule. The proliferative responses of its constituent peptides were almost completely subjugated in favour of junctional neo-determinants¹⁹. The present findings indicate that the differences in immunogenicity are likely to be attributed to the altered activity of the individual immunogenic peptide's abilities to recruit T-cells, which, in turn direct antibody response towards the neutralizing B-cell epitope(s) of interest.

Yet another possible reason for the failure of mini-RCP in eliciting neutralizing antibody response in spite of being constituted by linkage of the four independent neutralizing epitopic sequences is that the epitopes inserted might have lost the required antigenic and immunogenic properties in the newly

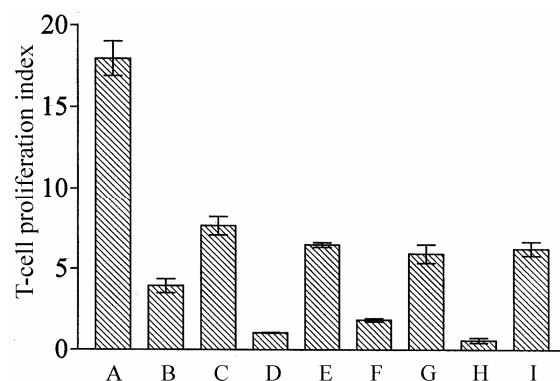


Fig. 5—Proliferation of T-lymphocytes from rats immunized with mini-RCP [Adult rats were immunized with 10 n mol of the antigen emulsified in FCA in the hind foot pads. After 15 days, the popliteal lymph node cells were collected and incubated with 10 n mol of either mini-RCP (A), ygc (B), YGC (C), ced (D), CED (E), gen (F), GEN (G), hac (H) or HAC (I). T-cell proliferation indices were determined after 4 days of culture. Values represent the mean \pm S. D. of triplicate cultures from 3 different experiments]

acquired conformation²⁰. In the crystal structure of native RCP²¹, these epitopes are located in the flexible loops/turns which comprise >60% of the protein length. It is well known that if the native protein is a flexible 3-dimensional spring, then local unfolding and refolding may occur all the time. The local unfolding of protein segments may permit the immunologic cross-reaction with anti-peptide antibodies because a flexible segment could assume many of the same conformations as the randomly folded free peptide(s)²².

It is pertinent to mention here that active immunization with a physical mixture of all the four individual neutralizing peptides of chicken RCP conferred protection from pregnancy in fertile rats and mice (unpublished observations). However, in mini-RCP, since the peptides are covalently-linked and without free N- and C-termini, there is a good chance of imposition of constraints on the flexibility of epitopic sequences. These findings strongly suggest that the prospects of covalently-linked epitope peptides need to be cautiously evaluated during the design and development of multiple epitope-based vaccines.

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