Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 311 (2003) 11–16
BBRC
www.elsevier.com/locate/ybbrc

Epitope mapping and evaluation of specificity of T-helper sites in four major antigenic peptides of chicken riboflavin carrier protein in outbred rats

Sarada Subramanian,[a,*] S. Andal,[b] Anjali A. Karande,[c] and P. Radhakantha Adiga[b]

[a] *Department of Neurochemistry, National Institute of Mental Health and Neurosciences, Bangalore 560 029, India*
[b] *Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560 012, India*
[c] *Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India*

Received 11 August 2003

Abstract

This paper reviews our studies on synthetic peptides spanning the major antigenic determinants of the chicken riboflavin carrier protein (RCP; 219 AA). These determinants are composed of residues 4–24 (YGC), 64–83 (CED), 130–147 (GEN), and 200–219 (HAC) and function as minivaccines in terms of eliciting anti-peptide antibodies which recognize the native protein and are particularly promising contraceptive vaccine candidates. We have used 15-residue synthetic peptides to define short sequences involved in interaction with antibody and with T-cells. We have mapped the boundaries of T-cell epitopes of these peptides in outbred rats by immunizing the animals with each peptide and assaying the popliteal lymph node cell proliferation against a series of overlapping synthetic 15-mers covering the entire length of the individual peptides. The peptides YGC, GEN, and HAC harboured a single T-cell epitope each whereas the peptide CED exhibited bimodal response possessing two epitopes, one at N-terminus and the other at the C-terminus. These studies provide insight into the way in which an immunogen is viewed by the immune system. In addition, preferential T-cell helper function for B cells recognizing unique determinants on the same molecule was demonstrated. This information helps in exploiting synthetic peptides in the construction of designer immunogens which have potential as candidate vaccines.

Keywords: Epitope mapping; Riboflavin carrier protein; Synthetic peptides; T-cell epitope

Riboflavin-carrier (RCP) or -binding protein (RfBP) is an evolutionarily conserved, monomeric globular protein [1–4]. This vitamin carrier was first identified in chicken egg and its functional importance in avian reproduction was demonstrated in a strain of chicken with a hereditary defect due to splice mutations in RCP gene [5] which led to the failure in hatching of eggs. Similarly, in mammals, immunoneutralization approaches unequivocally illustrated the requirement of RCP for successful completion of pregnancy in rodents [6] and sub-human primates [7]. Initially, these studies employed heterologous antigen viz., native chicken RCP to elicit immune response in mammals. It was soon realized that linearized, reduced, and carboxymethylated (RCM-RCP) is a preferred immunogen in terms of eliciting bioneutralizing antibodies to maternal RCP in rodents [8], thus highlighting the importance of linear antigenic determinants on chicken RCP sequence in immuno-interference with pregnancy progression. In efforts to decipher the location and characteristics of such epitopes, we have identified by PEPSCAN analysis six major sequential antigenic determinants with the core B-cell epitopes comprising of residues 10–17, 42–49, 68–83, 134–141, 172–179, and 200–207 in the 219 amino acid chain of chicken RCP [9]. Subsequently, all these individual sequences were synthesized as parts of larger oligopeptides (19- to 21-mers) with N- and C-terminal extensions to the 'core' B-cell epitopes to include potential T-cell determinants as per algorithm based

* Corresponding author. Fax: +91-80-656-4830.
E-mail address: sarada@nimhans.kar.nic.in (S. Subramanian).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.09.162

predictions such that they could function as minivaccines without conjugation to an extraneous carrier molecule. After confirming the presence of functional T-cell epitopes by T-lymphocyte proliferation assays in all the six sequences, the free peptides were then used for immunization of female mice, rats, and rabbits. They elicited very good immune response giving rise to both peptide-specific and RCP-crossreactive antibodies. When these actively immunized female animals were subjected to mating experiments, antibodies to four out of the six peptides interfered with pregnancy establishment in rodents indicating their potential as immunocontraceptive peptidyl vaccines [10,11]. These sequences included residues 4–23, 64–83, 130–147, and 200–219. These fragments were abbreviated as YGC, CED, GEN, and HAC peptides, respectively. The other two peptides comprising of residues 33–49 and 169–186 (abbreviated as CYA and CLQ, respectively), despite being immunodominant, failed to elicit neutralizing antibodies.

Peptide Number	Peptidyl sequence	Nomenclature
1.	GCLEGDTHKANPSPE	YGC-1
2.	CLEGDTHKANPSPEP	YGC-2
3.	LEGDTHKANPSPEPN	YGC-3
4.	EGDTHKANPSPEPNM	YGC-4
5.	GDTHKANPSPEPNMH	YGC-5
6.	DTHKANPSPEPNMHE	YGC-6
7.	YGCLEGDTHKANPSPEPNMHE	YGC
8.	AEDFTKKIECFYRAS	CED-7
9.	EDFTKKIECFYRASP	CED-8
10.	DFTKKIECFYRASPH	CED-9
11.	FTKKIECFYRASPHA	CED-10
12.	TKKIECFYRASPHAA	CED-11
13.	KKIECFYRASPHAAR	CED-12
14.	CEDFTKKIEAFYRASPHAAR	CED
15.	GENHCKSKAVPYSEM	GEN-13
16.	ENHCKSKAVPYSEMY	GEN-14
17.	NHCKSKAVPYSEMYA	GEN-15
18.	HCKSKAVPYSEMYAN	GEN-16
19.	GENHAKSKAVPYSEMYAN	GEN
20.	HACQKKLLKFEALQQ	HAC-17
21.	ACQKKLLKFEALQQE	HAC-18
22.	CQKKLLKFEALQQEE	HAC-19
23.	QKKLLKFEALQQEEG	HAC-20
24.	KKLLKFEALQQEEGE	HAC-21
25.	KLLKFEALQQEEGEE	HAC-22
26.	HACQKKLLKFEALQQEEGEE	HAC
27.	CYANFTEQLAHSPIIKV	CYA
28.	CLQMNKKDMVAIKHLLSE	CLQ

Fig. 1. Primary amino acid sequence of the peptides used in the present study; represented in single letter code.

It is widely acknowledged that epitope flanking residues influence the immunogenicity of T-cell epitopes [12–15] and also linear B-cell epitopes [16]. Hence, it is essential to detect and evaluate the importance of the amino acid residues recognized by T- and B-lymphocytes in order to induce an effective immune response. We have earlier identified and mapped the fine boundaries of the core B-cell epitopes in the four neutralizing peptides [9]. In the present study, we describe the fine mapping of the boundaries of T-cell epitopes of these four bioneutralizing peptides by using synthetic 15-mer peptides (Fig. 1) overlapping by one amino acid and covering the entire length of all the four immunodominant regions. Having ascertained the topographic relationships between B- and T-cell determinants in these individual peptide vaccines, it was of interest to investigate the potential of these T-cell epitopes to prime in vivo for antibody production directed against other dominant B-cell epitopes elucidated earlier. The data presented in this paper clearly support the view that the phenomenon of T–B cell reciprocity is restricted to constituent determinants of the individual peptide vaccines.

Materials and methods

Materials. Freund's complete and incomplete adjuvants (FCA/FIA), bovine serum albumin (BSA) (Fraction V), alkaline phosphatase conjugated goat anti-rabbit and anti-rat IgG, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St. Louis, USA). Phytohaemagglutinin, fetal bovine serum, and RPMI 1640 were procured from GIBCO laboratories (USA). [^{3}H]-Thymidine was obtained from Bhaba Atomic Research Center, Mumbai, India. High binding 96-well microtitre plates and tissue cultureware were purchased from Greiner Labortechnik (Germany). Chicken RCP was purified to homogeneity from chicken egg white [17]. All the synthetic peptides used in the present study were custom synthesized by Mimotopes Pty (Australia). Their individual purities (>95%) were established by reverse phase HPLC.

Animals. Wistar rats used for immunization were procured from the Institute's Central Animal Facility. These animals were housed in individual cages in a controlled environment of 12 h:12 h light–dark schedule and fed with pellet diet and water ad libitum.

T-cell proliferation assay. Lymphocyte stimulation assays were performed as described elsewhere [18]. Briefly, 3-month-old rats were injected with 100 μg of native RCP or 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 were prepared. About 4×10^5 lymph node cells were cultured in 200 μl RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine. Indicated amounts of the individual peptides were added as antigen source. Phytohaemagglutinin (5 μl/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% 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 incorporated radioactivity was measured in harvested cells in a β-scintillation counter. At least three repetitions of the assay were performed with comparable results. Data are represented 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 the cells cultured in the absence of the peptide. The specificity of the proliferative responses in each case was

ascertained by the lack of [^{3}H]-thymidine incorporation over and above that elicited in the absence of the cognate peptide during the pulse with [^{3}H]-thymidine by the other peptides at equivalent molar concentrations.

Evaluation of peptide-induced T-cell helper activity. The ability of peptide-primed rats to produce antibodies after challenge with RCP was determined following the protocol described elsewhere [19]. Briefly, groups of four rats were primed with 100 μg of peptide in FCA subcutaneously. Control rats were injected with FCA alone. Four weeks later, the rats were challenged with sub-optimal dose of RCP (20 μg) in FIA and sera were collected for antibody analysis on days 7 and 21 after booster challenge.

Characterization of the antisera. The immune recognition patterns of the pooled sera towards the free peptides as well as the native RCP or RCM-RCP were determined by direct ELISA [20]. Briefly, either 2 μg of peptide or 1 μg of the protein 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 diluted antisera. The antigen–antibody complex was visualized by employing alkaline phosphatase conjugated goat anti-rat IgG and *p*-nitrophenyl phosphate as the chromogen. The colour developed was monitored at 405 nm.

Results and discussion

Prediction of T-cell epitopes in peptidyl minivaccines

A number of approaches have been proposed for the identification of regions in protein or peptide sequences which can be recognized by T cells [21,22]. Empirical analysis of the regions recognized by T cells has shown that a high proportion of such sequences have the characteristic of being amphipathic helices. By using the computer algorithm AMPHI [21] which exploits the finding that, in general, many immunodominant T-helper sites have propensity to fold as amphipathic helices, we analyzed the peptidyl sequences to fold as amphipathic helices. The analysis revealed high scores with midpoints of the blocks localized to residues 54–74, 130–133, and 205–209 in CED, GEN, and HAC peptides, respectively (Fig. 2). Although the peptide YGC failed to exhibit amphipathic features, it did display a primary amino acid motif, predicted by the method of Rothbard and Taylor [22], to function as T-cell recognition site. This method defines a 4–5 amino acid sequence of a charged amino acid or glycine followed by two or three hydrophobic residues followed by a charged or polar residue to be a characteristic feature for T-cell recognition. This motif is present as GCLE within the sequence of YGC peptide (Fig. 2). Similar motifs have been found to be present in the other three peptides and they include the sequences DFTK and HAAR in CED peptide; EMYAN in GEN peptide, and HACQ, KLLK, and EALQ in HAC peptide.

YGC	YGCLEGDTHKANPSPEPNMHE
CED	**CE**DFTK**KIECF**YRCSPHAAR
GEN	**GENH**CKSKCVPYSEMYAN
HAC	HACQK**KLLKF**EALQQEEGEE

Fig. 2. Sequence of the antigenic peptides. The sequences underlined are the predicted T-cell epitopes as per Rothbard and Taylor's method. The sequences shown in bold are the mid-point blocks of the predicted AMPHI segments.

Analysis of the individual peptides for identification of the minimal T-cell epitopic sequences

In order to map the boundaries of 'core' T-cell epitopes in the immunodominant chicken RCP peptides, adult rats were immunized with individual parent YGC, CED, GEN, and HAC peptides. The popliteal lymph node cells were restimulated with either the corresponding parent peptide or individual 15-mer synthetic peptides corresponding to that parent peptide (10 μg–625 ng) for 4 days in culture. The amount of [^{3}H]-thymidine incorporated on day 5 is quantitated and enhanced levels are indicative of the proliferative responses (Fig. 3). Analysis of the results obtained with parent YGC peptide and its truncated 15-mers reveals that removal of the first two residues at the N-terminus did not affect the T-cell proliferating activity. However, in the peptide lacking the third residue, although exhibiting proliferative response, the extent of stimulation was significantly reduced. Further truncations led to total loss of recognition of the T-cell receptor/MHC-II binding activity. These results show that the minimal size of the epitope, recognized by the T-cells, composed of the residues ^{7}EGDTHKANPSP^{17} in the YGC peptide.

Critical examination of the various truncated peptides corresponding to the CED sequence reveals that all the peptides exhibited positive effect in terms of the induction of T-cell proliferative response. Among these, the peptide CED-9, which lacks N-terminal two residues and C-terminal three residues when compared to the parent peptide, showed least induction which increased with shift of the sequence either to the N-terminus or towards the C-terminus. From these results, it appears that the minimal recognition sequence is ^{66}DFTKKIECFYRCSPH^{80} and the flanking residues on either side enhance the stimulation brought about by this sequence. Alternatively, these results can be interpreted as presence of two T-cell epitopes within the parent CED sequence (one each at each terminus) matching with the predicted epitopes (by the method of Rothbard and Taylor) (Fig. 2) and the lesser induction brought about by CED-9 and CED-10 is attributed to the contributions made by partial epitopes from both the ends. A similar finding was observed in hen egg-white lysozyme where two distinct but overlapping T-cell determinants (residues 74–90 and 81–96) were present within a 23-amino acid region (residues 74–96)

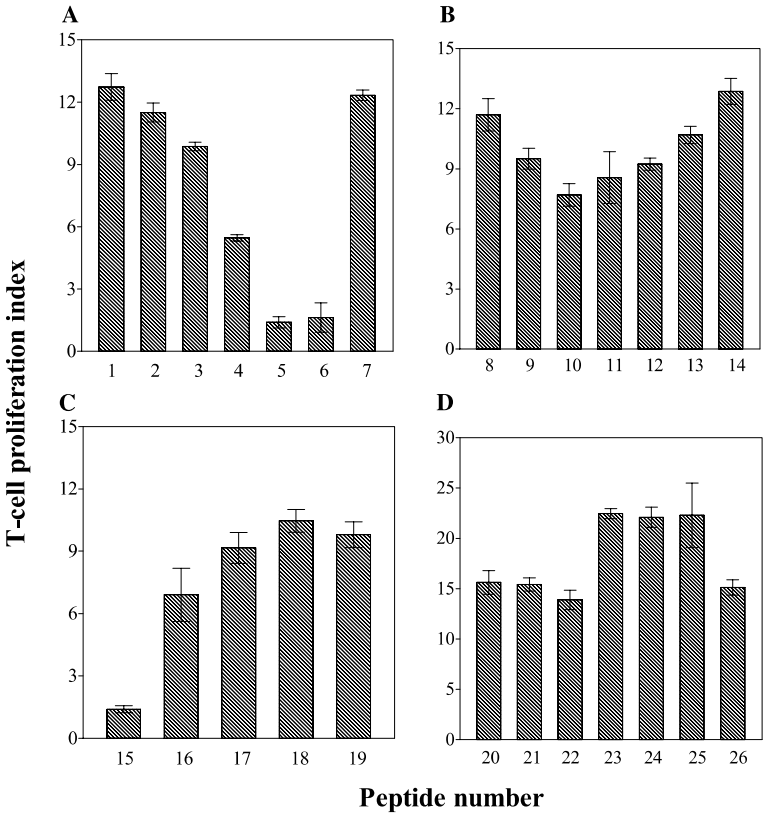

Fig. 3. Recognition of T-cell determinants. Popliteal lymph node cells from the peptide primed rats were stimulated with 10 nmol of truncated peptides corresponding to that parent peptide. Shown are the proliferative indices obtained with lymphocytes from rats primed with YGC (A), CED (B), GEN (C) or HAC (D). The number below each data point corresponds to the peptide number and its sequence described in Fig. 1. Values represent means ± SD of triplicate cultures from three different experiments.

[23]. The final proof as to which of these two hypotheses is valid awaits further study.

Among the GEN peptides, GEN-13 failed to stimulate the T-lymphocytes from GEN immunized rats in vitro. However, addition of a single tyrosine residue at the C-terminus regained the stimulating capacity with further addition of residues at this end bringing about only marginal further enhancement in the proliferative indices. It is clear from these results that tyrosine plays a crucial role in T-cell recognition and the minimal sequence can be narrowed down to ^{133}HCKSKCVPYS EMY^{145}.

Proliferative indices obtained with HAC peptides show two different levels of stimulation with the first three 15-mers and the parent HAC peptide exhibiting lesser stimulation in comparison to the latter three 15-mers which exerted 1.5-fold enhanced stimulation over the others. These results are in accordance with our earlier observations that within the HAC sequence, the peptides harbouring Cys residue show lesser induction than those which do not contain this residue. Since all the peptides could bring about the proliferation, it can be deduced that the minimal sequence common to all the truncated peptides (^{205}KLLKFEALQQ^{214}) is essential for binding.

In a reciprocal experiment, popliteal lymph node cells collected from RCP-immunized animals were in vitro

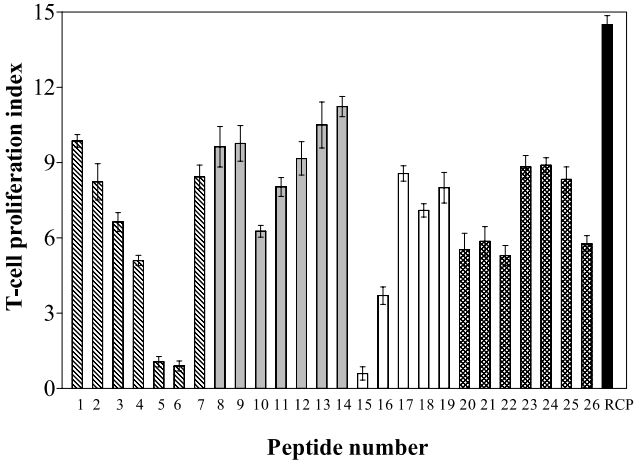

Fig. 4. Proliferation of T-lymphocytes from rats immunized with native RCP. Adult rats were injected with 10 nmol of the antigen emulsified in FCA in the hind foot pads. After 10 days, popliteal lymph node cells were collected and incubated with 10 nmol of the indicated peptides. T-cell proliferation indices were determined after 4 days of culture. The number below each data point corresponds to the peptide number and its sequence described in Fig. 1. Values represent means ± SD of triplicate cultures from three different experiments.

Table 1
Ability of chicken RCP peptides to prime T-cell function for antibody production

Priming peptide ($n = 4$)	Antiserum titres[a] against						
	RCP	YGC	CYA	CED	GEN	CLQ	HAC
YGC	2300	5000	–	–	100	–	–
CED	1200	–	–	3200	–	–	–
GEN	2700	–	–	–	4000	–	–
HAC	1200	–	–	–	–	–	850

[a] Titres measured on day 21 post-challenge are defined as the dilution of the antisera giving an O.D. value of 1.0 U at 405 nm.

restimulated with individual truncated peptides. The proliferative indices achieved (Fig. 4) were qualitatively similar to the profiles obtained with peptide-immunized groups. These findings strongly suggest that the native protein and peptides are immunologically processed in a similar manner.

Evaluation of peptide-induced T-cell helper activity

As shown in Table 1, priming with individual peptides resulted in antibody production specific for the determinant exclusively present within its sequence. The control rats which were primed with FCA alone and challenged with sub-optimal doses of the protein exhibited marginal response over the background levels with a titre of <100 dilution of the serum (data not shown). Since our earlier observations revealed the presence of at least two immunodominant yet non-neutralizing epitopes, it was of utmost importance to assess the specificity of T-cell epitopes within the neutralizing peptides since antibody production to the irrelevant determinants would be highly undesirable in view of an effective vaccine development. Earlier studies by Milich et al. [19] on hepatitis B surface antigen (HbsAg) have demonstrated that a dominant T-cell recognition site in pre-S(1) region (residues 12–21) can prime in vivo antibody production to multiple B-cell epitopes within the pre-S(2) and S regions as well. The findings of the present study rule out such a possibility and conclusively demonstrate the fine specificity of these epitopes.

We have recently demonstrated that substitutions in the flanking regions of core B- and T-cell epitopes in HAC sequence resulted in the modified peptide being more efficient in eliciting higher affinity, longer-lasting, RCP-crossreactive antibodies with consequent, more prolonged immunocontraceptive efficacy [24]. Fig. 5 summarizes the earlier mapped core B-cell epitopes and currently identified core T-cell epitopes of the four bioneutralizing peptides of chicken RCP. Further lines of investigations include engineering of these peptides outside the 'core' epitopes to enhance the immunogenicity and longevity of neutralizing antibody production leading to increased contraceptive efficacy of these minivaccines.

YGC	YGCLEGD**THKANPSP**EPNMHE
CED	CEDFTKKIECFY**RCSPHAAR**
GEN	GEN**HCKSKCVPY**SEMYAN
HAC	**HACQKKLL**KFEALQQEEGEE

Fig. 5. Localization of B- and T-cell epitopes within the chicken RCP peptides. Core B-cell epitopes are shown in bold letters. Minimal T-cell epitopes are underlined.

Acknowledgments

Financial assistance from the Department of Biotechnology, Government of India, is acknowledged. S.S. wishes to thank Department of Science and Technology, New Delhi, India for Young Scientist grant. P.R.A. is an INSA (Indian National Science Academy) Senior Scientist.

References

[1] P.R. Adiga, Riboflavin carrier protein in reproduction, in: K. Dakshinamurti (Ed.), Vitamin Receptors, Cambridge University Press, Cambridge, 1994, pp. 137–176.
[2] K.B. Storey, M.E. Dent, J.M. Storey, Gene expression during estivation in spadefoot toads *Scaphiopus couchii* : Upregulation of riboflavin binding protein in liver, J. Exp. Med. 284 (1999) 325–333.
[3] S. Hamajima, S. Onu, Sequence of a cDNA encoding turtle riboflavin-binding protein: comparison with avian riboflavin-binding protein, Gene 164 (1995) 279–282.
[4] S. Subramanian, P.R. Adiga, Immunological relatedness of chicken and human riboflavin carrier protein, Biochem. Biophys. Res. Commun. 262 (1999) 539–544.
[5] I. MacLachlan, J. Nimpf, H.B. White, W.J. Schneider, Riboflavinuria in the rd chicken: 5′-splice site mutation in the gene for riboflavin binding protein, J. Biol. Chem. 268 (1993) 232221–232226.
[6] C.V.R. Murty, P.R. Adiga, Pregnancy suppression by active immunization against gestation-specific riboflavin carrier protein, Science 216 (1982) 191–193.
[7] P.B. Seshagiri, P.R. Adiga, Pregnancy suppression in bonnet monkey by active immunization with chicken riboflavin carrier protein, J. Reprod. Immunol. 12 (1987) 93–107.
[8] A.A. Karande, P.R. Adiga, Early pregnancy termination in rats immunized with denatured chicken riboflavin carrier protein, Indian J. Biochem. Biophys. 28 (1991) 476–480.

[9] S. Subramanian, P.R. Adiga, Identification and mapping of linear antigenic determinants of chicken riboflavin carrier protein, Biochim. Biophys. Acta 1429 (1998) 74–82.
[10] S. Subramanian, A.A. Karande, P.R. Adiga, Immunocontraceptive efficacy of synthetic peptides corresponding to major antigenic determinants of chicken riboflavin carrier protein in the female rats, Am. J. Reprod. Immunol. 44 (2000) 184–191.
[11] S. Subramanian, A.A. Karande, P.R. Adiga, Immunocontraceptive potential of major antigenic determinants of chicken riboflavin carrier protein in the female rats, Vaccine 19 (2000) 1172–1179.
[12] M.S. Vacchio, J.A. Berzofsky, U. Krzych, J.A. Smith, R.J. Hodes, A. Finnegan, Sequences outside a minimal immunodominant site exert negative effects on recognition by staphylococcal nuclease-specific T cell clones, J. Immunol. 143 (1989) 2814–2819.
[13] G. Gammon, H.M. Geysen, R.J. Apple, E. Pickett, M. Palmer, A. Ametani, E.E. Sercarz, T cell determinant structure: cores and determinant envelopes in three mouse major histocompatibility complex haplotypes, J. Exp. Med. 173 (1991) 609–617.
[14] R. Janssen, M. Wauben, R. Zee van der, M. de Gast, J. Tommassen, Influence of amino acids of a carrier protein flanking an inserted T cell determinant on T cell stimulation, Int. Immunol. 6 (1994) 1187–1193.
[15] E. Roman, D.P. Harris, S. Jurcevic, J. Ivanyi, C. Moreno, H-2-associated effects of flanking residues on the recognition of a permissive mycobacterial T cell epitope, Immunology 86 (1995) 183–189.
[16] C. Leclerc, P. Martineau, A. Charbit, M.R. Lo, E. Deriaud, M. Hofnung, Immunodominance of a recombinant T cell epitope depends on its molecular environment, Mol. Immunol. 30 (1993) 1561–1572.
[17] U.S. Murthy, P.R. Adiga, Riboflavin binding protein of hen's egg: purification and radioimmunoassay, Indian J. Biochem. Biophys. 14 (1977) 118–124.
[18] S. Mouritsen, M. Meldal, B. Rubin, A. Holm, O. Werdelin, The T-lymphocyte proliferative response to synthetic peptide antigens of defined secondary structure, Scand. J. Immunol. 30 (1989) 723–730.
[19] D.R. Milich, A. McLachlan, A. Moriarty, G.B. Thornton, A single 10-residue pre-S(1) peptide can prime T cell help for antibody production to multiple epitopes within the pre-S(1), pre-S(2), and S regions of HbsAg, J. Immunol. 138 (1987) 4457–4465.
[20] A.A. Karande, N.K. Velu, P.R. Adiga, A monoclonal antibody recognizing the C-terminal region of chicken egg white riboflavin carrier protein terminates early pregnancy in mice, Mol. Immunol. 28 (1991) 471–478.
[21] H. Margalit, J.L. Spouge, J.L. Cornett, K.B. Cease, C. De Lisi, J.A. Berzofsky, Prediction of immunodominant helper T-cell antigenic sites from the primary sequence, J. Immunol. 138 (1987) 2213–2219.
[22] J.B. Rothbard, W.R. Taylor, A sequence pattern common to T-cell epitopes, EMBO J. 7 (1988) 93–100.
[23] N. Shastri, G. Gammon, S. Horvath, A. Miller, E.E. Sercarz, The choice between two distinct T-cell determinants within a 23-amino acid region of lysozyme depends on their structural context, J. Imunol. 137 (1986) 911–915.
[24] S. Subramanian, A.A. Karande, P.R. Adiga, 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, Biochem. Biophys. Res. Commun. 287 (2001) 236–243.