

# *v-src* oncogene-specific carboxy-terminal peptide is immunoprotective against Rous sarcoma growth in chickens with MHC class I allele B-F12

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## Abstract

$B^{12}$  haplotype of the inbred chicken line CB ( $B^{12}/B^{12}$ ) contains, like the bulk of chicken *MHC(B)* haplotypes, only a single dominantly expressed class I molecule (B-F). The peptide binding motifs for this major B-F12 molecule in chickens of Rous sarcoma regressor line CB ( $B^{12}/B^{12}$ ) have been determined. Using stringent and relaxed motifs, several peptides were found in the *v-src* molecule of the PR-RSV-C, but most of these peptides are identical with that of endogenous *c-src*. Only the *v-src* C-tail peptide<sub>517–524</sub> (LPACVLEV) contains critical anchor amino acids (valine at positions 5 and 8) and shows a sequence different from the corresponding *c-src* peptide. This *v-src* C-tail peptide up-regulates expression of the B-F12 class I molecule on PBL, as assessed by FACS analysis, and stimulates T cell proliferation in a [<sup>3</sup>H]thymidine uptake assay. A protective effect of the immune response to LPACVLEV against RSV challenge was demonstrated in CB ( $B^{12}/B^{12}$ ) chickens immunised with peptides encapsulated in liposomes.

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## 1. Introduction

Rous sarcoma virus (RSV) tumorigenesis is mediated by the oncogene *v-src* [1–3]. Previous studies with Prague congenic lines have unambiguously established that the ability to regress sarcomas induced by Rous sarcoma virus or the LTR, *v-src*, LTR proviral DNA is closely associated with the *MHC(B)* of the chicken [4,5]. The immune-based mechanism of tumour regression was demonstrated by both in vivo and in vitro assays [6,7]. Similar results were obtained also in other experimental systems [8–10].

The peptide binding motifs for class I (B-F) molecules of the *B* haplotypes of Rous sarcoma growth resistant and susceptible chicken lines have been determined. Each *MHC(B)* haplotype comprises, in contrast to the well-studied mammalian MHCs, only one dominantly expressed class I molecule. Furthermore, the peptide binding specificity corresponds precisely to the observed ability of RSV-induced tumour regression [11–14]. Here, we present the data ver-

ifying the theoretical assumptions using one of the Prague inbred line CB ( $B^{12}/B^{12}$ ) and synthetic peptides from the *v-src* gene protein, predicted to fit class I motif of the  $B^{12}$  allele, as immunogen.

The general view has been that the MHC class I molecules exclusively present peptides from endogenous proteins, whereas the class II molecules present exogenous antigens. A great deal of evidence, however, demonstrates that a subset of APC can also acquire and present exogenous antigens via class I molecules (for review see [15,16]). This exogenous pathway of antigen presentation provides an opportunity to prime CTL responses by protein-based vaccines when appropriately delivered. In our experiments, by far the best results were obtained with *v-src*-derived peptides encapsulated in liposomes.

## 2. Materials and methods

### 2.1. Animals, nomenclature

Chickens of the Prague inbred line CB ( $B^{12}/B^{12}$ ) [17,18] free of avian leukosis viruses were used for the experiments.

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The designation of the *MHC(B)* alleles conforms to the internationally adopted nomenclature [19].

## 2.2. *MHC-peptide binding assay (according to the unpublished method developed by J. Kaufman)*

PBL were isolated from heparinized blood by slow speed centrifugation. A total of  $5 \times 10^5$  cells per well in DMEM (GIBCO) supplemented with 2 mg/ml bovine serum albumin (BSA), penicillin (100 U/ml) and streptomycin (100 µg/ml) were cultured with peptides (100 µg/ml) in a 96-well U-bottom microtiter plate (final volume 100 µl per well) for 16 h in an incubator at 40 °C, 5% CO<sub>2</sub> and 100% humidity. After three washings, cells were stained with 50 µl of monoclonal antibody (F21/21) against β<sub>2</sub>M, washed again and incubated with 50 µl of a 1:100 dilution of a FITC-conjugated rabbit anti-mouse IgG (F261, DAKO, Denmark). After three washings, cells were suspended in 150 µl PBS with 10 mg/ml BSA, 0.1% NaN<sub>3</sub> and 5 µg/ml propidium iodide. Five thousand of propidium iodide negative cells per sample were analysed in flow cytometry using a FACScan (Becton Dickinson).

## 2.3. *Preparation of peptides in different delivery systems*

Peptides dissolved in PBS were emulsified in incomplete Freund's adjuvant (IFA) by repeated passing through a syringe. Peptides in microspheres were prepared according Kofler et al. [20]. Liposomes entrapping peptides were prepared according New [21] and Lipford et al. [22]. Briefly, 18 mg L-α-phosphatidylcholine, 2 mg L-α-phosphatidyl-DL-glycerol and 5 mg cholesterol (all components from Sigma) were suspended in 5 ml chloroform and rotary evaporated under reduced pressure until a thin lipid film formed on the flask wall. Residual chloroform was removed by vacuum desiccation. Three milligrams of peptide were dissolved in 1 ml of PBS containing 0.4 mg QuilA and 1 mM 2-mercaptoethanol. This solution was added to the dried lipids and slowly shaken until the lipids were re-suspended and then equilibrated 30 min at room temperature. Peptides incorporation efficacy was 29% for *v-src*-derived peptide<sub>200–207</sub> (KHYKIYKL) and 19% for *v-src* C-tail peptide<sub>517–524</sub> (LPACVLEV) as determined with the help of <sup>125</sup>I-labelled peptides (Amersham, UK).

## 2.4. *Peptide-specific T cell proliferation*

CB chickens were injected twice i.m. at 2 weeks intervals with a dose of 150 µg per chicken per injection of peptide prepared in IFA, microspheres or liposomes (see earlier). Five days after the last injection 10<sup>6</sup> PBL per well (96-well U-bottom microtiter plate) were cultured in Iscove's DMEM optimised for chicken cells [23] for 48 h with the peptide, ConA as a positive control or in medium only as a negative control. The amount of peptide was 5 µg per well in total volume of 50 µl and for ConA 4 µg per well in a total volume

100 µl. Cultures were pulsed with [<sup>3</sup>H]thymidine (1 µCi per well) for the last 6 h.

## 2.5. *Immunisation, RSV challenge, tumour monitoring*

CB chickens were immunised twice at the age of 10 and 12 weeks with 100 µg peptide in 0.2 ml of liposomes per injection. Injections were done i.m. into the pectoral muscle. Some chickens received two additional injections i.d. into the right wing web at the age of 11 and 13 weeks. All chickens were challenged at the age of 14 weeks with the Prague strain of RSV (PR-RSV-C), obtained and titrated on leukosis free CEFs [5]. Chickens were inoculated with a dose of virus corresponding to 100 focus forming units (FFU) in 0.1 ml of Iscove's DMEM, s.c. into the left wing web. The size of tumours was measured by calculating the area (mm<sup>2</sup>) of the tumour prominent from the wing web [5].

## 2.6. *Statistical analysis*

We used the statistical program GraphPad Prism™ version 2.0 (Intuitive Software for Science, San Diego, CA) for statistical analysis.

## 3. Results

### 3.1. *Up-regulation of MHC(B) class I (B-F) molecule expression on PBL by synthetic peptides*

Altogether 13 peptides from the *v-src* gene of the Prague strain of Rous sarcoma virus were predicted to fit the binding motif of the B-F12 class I molecule dominantly expressed within the B<sup>12</sup> haplotype of the inbred chicken line CB (B<sup>12</sup>/B<sup>12</sup>). All these synthetic peptides were tested for actual binding to the B-F12 class I molecule by incubation with PBL and FACS analysis. Table 1 shows the results obtained with four representative peptides. Only the *v-src* C-tail peptide<sub>517–524</sub> binds B-F12 class I molecule with sufficient affinity to enhance significantly its expression on PBL under the conditions of the in vitro test used. Two other peptides with stringent motifs are less effective. The octapeptide GENLVCKV is more effective than the nonapeptide LAG-GVTTFV but this difference is not significant. The lowest effect was seen with peptides harbouring relaxed motif like the KHYKIYKL. LPACVLEV and KHYKIYKL peptides were further analysed because of their potential immunogenicity in chickens due to sequence differences from the endogenous *c-src* sequence (Table 1).

### 3.2. *Peptide-specific immunity in chickens of the CB (B<sup>12</sup>/B<sup>12</sup>) line*

Chickens of the CB (B<sup>12</sup>/B<sup>12</sup>) line were immunised with synthetic peptides using different delivery methods.

Table 1

Up-regulation of MHC class I (*B-F*) expression on PBL of the CB ( $B^{12}/B^{12}$ ) line by synthetic peptides with predicted *B-F*<sup>12</sup> motif found in the *v-src* gene of PR-RSV-C

Treatment with peptide: location in <i>v-src</i>	Sequence <sup>a</sup>	Stringency	Mean fluorescent intensity (MFI) ± S.D. <sup>b</sup>
SH3 domain (79–87)	LAGG <u>V</u> TT <u>F</u> V	Stringent	663.9 ± 66.3 a
Kinase domain (395–402)	GEN <u>L</u> V <u>C</u> KY	Stringent	691.2 ± 61.9 a
C-tail (517–524)	<b>LPACV</b> <u>L</u> <b>E</b> <u>V</u>	Stringent	814.7 ± 101.8 b
SH2 domain (200–207)	KHY <u>K</u> I <u>Y</u> K <u>L</u>	Relaxed	643.9 ± 76.5 a
None			606.2 ± 58.2 a

<sup>a</sup> Amino acids different from the corresponding sequence in the endogenous *c-src* are printed in bold latter. Underlined letters represent anchor amino acids in a given peptide.

<sup>b</sup> MFI of PBL was measured after 16 h incubation with or without 100 µg/ml of peptides. Cells were then stained with a monoclonal anti-β<sub>2</sub>M antibody, and FITC-conjugated rabbit anti-mouse IgG and analysed by flow cytometry (for details see Section 2). Values represent three independent experiments, each done in triplets, with essentially the same results. Values not sharing the same letter are significantly different ( $P > 0.05$ ) Student's *t*-test.

Table 2 shows that *v-src* C-tail peptide<sub>517–524</sub>, shown to bind effectively the B-F12 molecule (see earlier), can also induce, when appropriately delivered, an immune response in chickens. Encapsulation in liposomes is the most effective, while the peptide delivery in incomplete Freund's adjuvant does not induce significant immunity in CB chickens. This peptide-specific immunity can be demonstrated by re-stimulation of PBL from immunised donors *in vitro*, as measured by incorporation of [<sup>3</sup>H]thymidine. The second potentially immunogenic *v-src*-derived peptide<sub>200–207</sub> does not induce an immune response detectable *in vitro*. Thus, it is reasonable to consider the C-tail peptide<sub>517–524</sub> as B-F12 restricted immunodominant peptide of the *v-src* protein.

### 3.3. Protective effect of peptide immunisation on RSV-induced tumour growth in CB ( $B^{12}/B^{12}$ ) chickens

Results of two independent experiments with CB ( $B^{12}/B^{12}$ ) chickens immunised with liposome entrapped *v-src* peptides LPACVLEV and KHYKIYKL are shown in Table 3. Diminution of the rate of RSV-induced tumour

growth is evident in chickens immunised with both peptides but only the group of chickens immunised four times with the C-tail peptide<sub>517–524</sub> is partially protected against induction of tumours. The incidence is 55.6% (5/9), while all other groups developed tumours with 100% incidence. It should be stated that the two peptides used are entrapped by liposomes with different efficiency which is lower for LPACVLEV (19%) than for KHYKIYKL (29%) (Fig. 1).

## 4. Discussion

It is nearly two decades ago that the gene responsible for regression of RSV-induced tumours was located into the *B-F/L* region of the chicken *MHC(B)*. This assumption resulted from immunogenetic analysis of the Prague recombinant congenic lines [24–26]. The  $B^{12}$  haplotype of regressor line CB, the first *MHC* haplotype analysed at the molecular level in chickens [27], has been shown to contain only one dominantly expressed class I allele in the *B-F/L* region [11]. Furthermore, the peptide binding specificity of the B-F12 could be used to explain the observed  $B^{12}$  association with

Table 2

*In vitro* proliferation of PBL from the CB ( $B^{12}/B^{12}$ ) chicken immunised with *v-src* peptides.

Immunisation with:	Adjuvant used	In vitro restimulation with:	Stimulation index (SI) ± SD	No.chicks with SI>2/No.chicks tested	
LPACVLEV	IFA	LPACVLEV	2.01±1.19	2/8	
		Microspheres	2.30±0.40		
		Liposomes	3.36±1.22		
	Liposomes	IFA	KHYKIYKL	1.60±0.40	2/8
		Microspheres		1.54±0.40	2/9
		Liposomes		1.47±0.40	0/9
KHYKIYKL	Liposomes	KHYKIYKL	0.83±0.07	0/5	
		LPACVLEV	0.89±0.21	0/5	
	Liposomes	LPACVLEV	2.09±1.67	5/16	
		KHYKIYKL	1.19±0.38	0/8	

CB chickens were injected twice *i.m.* and 5 days after the second injection PBL proliferation was measured as [<sup>3</sup>H]thymidine incorporation. Data represent mean stimulation index (cpm of test sample/cpm of medium control) of individual PBL cultures set in triplets for each chicken. For details see Section 2. \* $P > 0.05$ ; \*\* $P > 0.01$ ; \*\*\* $P > 0.001$ ; n.s.: not significant (Student's *t*-test).

Table 3

Protective effect of peptide immunisation<sup>a</sup> on RSV-induced tumour growth in CB (*B12/B12*) chickens (mean tumour size in mm<sup>2</sup> ± S.E.M.)

Experiment A	Days past injection of RSV					tumour incidence	
	12	14	17	21	24		28
LPACVLEV 2 injections	35.4±10.4	58.7±11.9	62.1±10.9	20.0±8.1	4.1±1.9	3.1±3.1	15/15
KHYKIYKL 2 injections	29.7±7.9	64.0±9.5	77.2±13.7	45.1±12.5	18.8±10.2	17.6±8.3	15/15
- (PBS) 2 injections	57.1±11.2	104.5±10.9	143.2±15.9	107.1±13.7	42.4±11.3	20.5±11.4	15/15
			n.s.	*	*		
			**	**	*		
Experiment B	Days past injection of RSV					tumour incidence	
	12	15	19	23	29		
LPACVLEV 4 injections	4.5±2.1	9.2±3.2	19.0±10.3	8.9±6.5	4.2±1.9	5/9	
LPACVLEV 2 injections	49.0±7.7	62.2±8.5	62.9±21.1	23.7±15.3	13.6±7.9	10/10	
KHYKIYKL 4 injections	41.9±13.5	86.2±6.9	80.8±12.8	39.4±8.2	1.6±1.5	9/9	
KHYKIYKL 2 injections	63.2±12.5	114.1±16.1	134.3±19.4	33.8±5.3	23.3±15.4	9/9	
- (PBS) 4 injections	105.4±13.7	119.0±10.6	120.6±52.7	116.2±98.9	77.8±42.5	5/5	
			*	n.s.			
			***	*	**		

<sup>a</sup> CB chickens were immunised twice at the age of 10 and 12 weeks. Peptide dose was 100 µg per chick per injection in 0.2 ml of liposomes. Injections were done i.m. into the pectoral muscle. Some chickens obtained two additional i.d. injections at the age of 11 and 13 weeks. All chickens were challenged at the age of 14 weeks with 100 FFU of PR-RSV-C.

resistance to progressive growth of Rous sarcomas. It is noteworthy that the only *v-src* peptide, fitting to the class I motif of the *B-F12* allele and simultaneously with totally different sequence from that of cellular homologues *c-src* (Table 1), was found in the C-tail of the *src* molecule. Mutations in this part of the *src* molecule with critical auto-regulatory residue Y527 are frequent and the loss of this phosphorylation site is responsible for the transforming capacity of *v-src* oncogenes [3]. *C-src* activation is a common event in human colon cancer [28,29]. Naturally occurring mutants at the carboxy-terminal (C-terminal) regulatory region, with truncation of Y530 (human equivalent of Y527 in chicken *c-src*), appeared to be responsible for the remarkable per-

centage of advanced metastasising colon cancer [30]. Of great importance would be that mutations accumulated in the C-tail of *src* molecule, not only change the cellular protooncogene to a transforming oncogene, but also may create antigenic motifs fitting to some *MHC* alleles, thus targeting these new oncogenes to the CTL response. In the present study, we have shown that the peptide LPACVLEV found in the C-tail (amino acids 517–524) of *v-src* gene of PR-strain of RSV can bind to the *B-F12* allele and stimulates both an in vitro and in vivo response against RSV-induced tumours in the CB(*B12/B12*) chickens (Tables 1 and 2). It has been described that sequence variation in *src* genes may influence tumour growth pattern and even metastasis formation

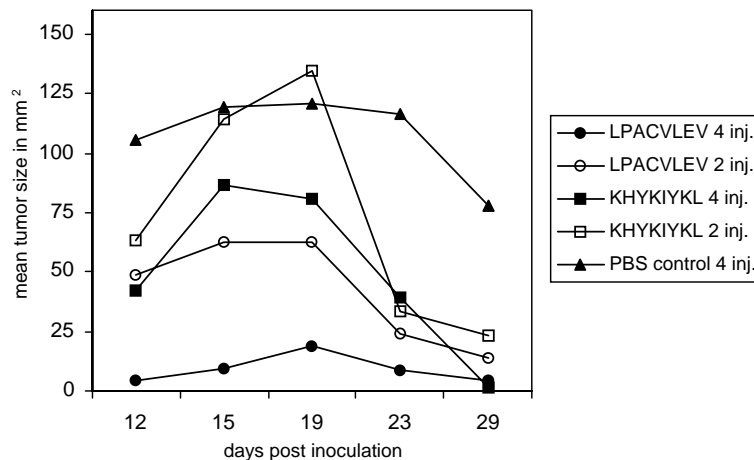


Fig. 1. Protective effect of peptide immunisation on RSV-induced tumour growth in CB (*B12/B12*) chickens mean tumour size (mm<sup>2</sup>) ± S.E.M.

in chickens [31,32] and hamsters [33]. Both alteration of *src* kinase activity and changes of antigenicity might contribute to the observed differences. It is interesting in this context, that *v-src* genes, derived from SR strain of RSV by Tatosyan et al. [33] in a hamster cell line, harbour mutations abolishing anchor positions—valine (V)—of the *B-F*<sup>12</sup> binding motif LPACVLEV. Indeed, these *v-src* genes-induced progressive tumour growth with frequent metastasis formation, when injected into the CB(*B*<sup>12</sup>/*B*<sup>12</sup>) chickens in an appropriate LTR, *v-src*, LTR DNA construct (our own unpublished results). Another strain of RSV-BH also induces progressively growing tumours in CB chickens [34]. In this case, negatively charged glutamic acid (E)—next to the anchor position of LPACVLEV—is replaced by positively charged lysine (K). The significance of this change awaits further analysis. Third indirect evidence, supporting the decisive role of C-tail sequence of *v-src* gene product for the outcome of tumour growth in CB chickens, came from experiments with variant *c-src* genes carrying point mutations. Tumours induced by *c-src* constructs with the only change of tyrosine (Y) 527 to phenylalanine (F) grew again rather progressively in CB chickens in comparison with *v-src* constructs ([35] and unpublished results).

It has been shown previously that peptides eluted from class I molecules of particular *MHC(B)* haplotype specifically raised the total number of class I molecules on appropriate PBL [12]. *B*<sup>12</sup> haplotype belongs to that strains with naturally high level of expression of major *B-F* (class I) allele, thus leaving relatively narrow scope for even up-regulation by synthetic peptides. Nevertheless, the effect of *v-src* C-tail peptide<sub>517–524</sub> is clear and highly reproducible.

The avian immune system works similarly to that of well studied mammals, but there are also significant differences. One drawback of the chicken system is that in vitro measured parameters of cell proliferation are always lower than in mammals. In chickens, it is also not possible to isolate antigen-specific lymphocytes from the draining lymph nodes where the frequency of those cells should be higher than in spleen or peripheral blood. Thus, stimulation indexes (<sup>3</sup>H)thymidine incorporation) obtained in our experiments are not directly comparable with usual values in mice. Again, only *v-src* C-tail peptide gives significant results when tested for T cell re-stimulation in vitro (see Table 2) and is also superior in protection against RSV-induced tumour growth in vivo. The second potentially immunogenic peptide tested, KHYKIYKL, which gave largely negative results in vitro, shows only slight protective effect when tested in vivo.

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