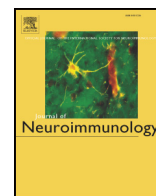




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Eluding anaphylaxis allows peptide-specific prevention of the relapsing stage of experimental autoimmune encephalomyelitis[☆]

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ABSTRACT

We have used a peptide derived from *Acanthamoeba castellanii* (ACA) to treat the relapsing phase of EAE that develops in SJL mice following immunization with the PLP 139–151 peptide. The native sequence of the ACA 81–95 peptide that shares key residues with the PLP 139–151 peptide is weakly encephalitogenic in SJL mice but is not recognized by antiserum from SJL mice immunized with PLP 139–151. A single amino acid change to the ACA 81–95 peptide sequence significantly enhanced its encephalitogenicity. When administered to SJL mice as a non-linear peptide octamer, the modified ACA peptide prevented relapsing episodes of EAE in SJL mice previously immunized with the PLP 139–151 encephalitogenic peptide.

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1. Introduction

The inbred SJL mouse strain develops a relapsing/remitting course of EAE (rEAE) following immunization with different myelin-derived proteins, or specific encephalitogenic peptides derived from these proteins (Amor et al., 1994; Bhardwaj et al., 1994; Kaushansky et al., 2007, 2008). Using this model, peptide specific therapies have been developed to successfully prevent and, in some cases, treat established disease (Elliott et al., 1996; Leadbetter et al., 1998; Smith and Miller, 2006). However the concomitant humoral response to the encephalitogen used to induce disease can prompt an anaphylactic response to the therapeutic agent, not only preventing a detailed analysis of the clinical effect that specific peptide therapy might render at the relapse stage of disease, but also perhaps more importantly placing the recipient of the therapeutic agent at significant risk. For example, it has been reported that the humoral response that develops following sensitization to the proteolipid protein (PLP) 139–151 peptide encephalitogen leads to anaphylaxis when soluble PLP 139–151 peptide is subsequently administered to these mice (Pedotti et al., 2001; C.E. Smith et al., 2005). We have also noted the development of anaphylaxis in SJL mice treated with nonlinear peptide octamers of this encephalitogen to modify the course of EAE in mice following immunization with the encephalitogenic PLP 139–151 peptide monomer, thus preventing an analysis of the

therapeutic effect of the octameric peptide at the relapsing stage of disease (Wegmann et al., 2008).

In order to assess the treatment effect using soluble peptides in mice following recovery from primary diseases, without the concern for anaphylaxis, we have evaluated the PLP peptide 139–151 molecular mimic derived from the protozoan *Acanthamoeba castellanii* (ACA). This peptide is reported to be encephalitogenic in SJL mice and contains the TCR and MHC contact residues in common with the PLP 139–151 encephalitogenic peptide that has been determined for SJL mice (Massilamany et al., 2010). Other amino acids in the ACA peptide are distinct from the PLP 139–151 peptide sequence providing a natural modification to the PLP 139–151 peptide encephalitogen.

We synthesized the ACA peptide and when used as an immunogen, also found it to be weakly encephalitogenic in SJL mice. We also discovered that the ACA peptide was not recognized by antiserum obtained from SJL mice previously immunized with the PLP 139–151 encephalitogenic peptide. In subsequent studies we substituted amino acids in the native ACA sequence resulting in significant improvement in its encephalitogenicity. Although these modifications promoted ACA encephalitogenicity they did not result in recognition of the altered peptide by antiserum reactive with the PLP 139–151 peptide. Subsequently we evaluated the native ACA and one modified peptide as nonlinear peptide octamers to prevent the relapse phase of EAE in SJL mice immunized with the PLP 139–151 encephalitogenic peptide. We found that although both peptide octamers had some impact on the development of the relapse phase of disease the modified ACA peptide octamer with the greater intrinsic encephalitogenicity was the most effective therapeutic when administered in a soluble form to mice that had

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recovered from their primary course of EAE induced following immunization with the PLP 139–151 encephalitogenic peptide. We further show in this report that this modified ACA octamer prevented the development of rEAE in mice treated with this octamer, and the prevention of the relapsing stage of disease occurred with no signs of anaphylaxis during treatment with the modified ACA peptide-octamer.

2. Materials and methods

2.1. Animals

Female SJL mice (4–6 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions at the Veterans Affairs Medical Center Animal Care Facility (Portland, OR), according to institutional guidelines and were acclimated for at least two weeks prior to experimental use. 5B6 TCR transgenic mice that respond to the PLP 139–151 peptide were obtained from a colony maintained within the animal care facility. All animal studies were reviewed and approved by the IACUC of the VAMC, Portland, OR.

2.2. Antigens

The PLP 139–151 peptide (amino acid 140 C to S substitution, amino acid sequence HSLGKWLGHDPDKF) was commercially prepared by Genscript (Piscataway, NJ) and supplied at 90% purity. All MAP peptide octamers were synthesized on a Protein Technologies PS3 peptide synthesizer using standard F-moc chemistry and a double coupling procedure. MAP peptides were prepared by the sequential addition of amino acid residues to an eight-branched MAP resin (#05–24–0151, EMD chemicals San Diego, CA). The MAP peptides were designed to contain the peptide sequence with added lysine spacers when required to enhance solubility (Brokx et al., 2002). The MAP synthesis was started with two glycine residues as spacers for all peptide octamers used in this study. The sequence of the PLP_{139–151}MAP peptide with the glycine spacers added is (HSLGKWLGHDPDKFGG)₈-K₄-K₂-K-beta-A. The sequence of the ACA_{81–95}MAP is (KRGYFLKWLGHDPNVSKGG)₈-K₄-K₂-K-beta-A and the modified ACA_{81–95}MAP with a leucine to glycine substitution at position 86 is (KRGYFLGKWLGHDPNVSKGG)₈-K₄-K₂-K-beta-A. To improve the solubility of all ACA_{81–95}MAP derived peptides, lysines (K) were added to both the N-termini and di-glycine spacer of each octamer branch. Other peptide modifications to the ACA peptide sequence are as indicated in the text. The PLP 141–149 minimal peptide sequence is (LGKWLGHDPDGG)₈-K₄-K₂-K-beta-A. The sequence of the OSP_{55–71}MAP peptide is (KKDCVMATGLYHCKPLVDIKGG)₈-K₄-K₂-K-beta-A. To improve the solubility of OSP_{55–71}MAP OSP two lysines were added to the N-termini and a single lysine added to the di-glycine spacer of each octamer branch. All peptides were cleaved with a mixture of trifluoroacetic acid (80%), H₂O (8%), 1,2 Ethanedithiol (4%) and Thioanisole (8%) (Sigma, St. Louis, MO). The cleavage reaction was allowed to proceed from 1.5 to 2 h. After cleavage the peptides were purified from the cleavage byproducts by washing 3 times in tert-butyl methyl ether. The peptide pellets are dissolved in a 1:1 mixture of water:acetonitrile, frozen and lyophilized for long-term storage.

The resulting MAP products were assessed for purity by a commercial service (AAA Laboratories, Corvallis, OR). Because of the nature of the MAP octamers, the best indication of a successful synthesis is obtained from an analysis of their complete amino acid composition and then comparing the ratios of amino acid content to the fixed lysine content that constitutes the resin used as the basis of the MAP synthesis. Using the double coupling method for synthesis of the MAP peptides, we found that following cleavage from the resin the determined amino acid content was typically at least 90% of theoretical.

2.3. Induction of active EAE

EAE was induced in 9 to 12 week-old female SJL mice by the subcutaneous injection of encephalitogenic peptide (150–200 µg) emulsified in CFA. The injection volume of 0.2 ml was distributed equally between four sites on the flank. The CFA contained 2 mg/ml *Mycobacterium tuberculosis* strain H37Ra (Difco, Detroit, MI). This immunization protocol was also used to determine the existence of memory responses retained in mice following treatment with MAP peptides.

2.4. Scoring of clinical paralysis

Clinical signs of actively induced EAE in SJL mice typically consist of hind limb weakness with limited forelimb involvement. However, animals that develop the most severe clinical disease signs develop both hind and forelimb paralysis. Degrees of hind limb and forelimb weaknesses were assessed as described below and as previously outlined (Whitham et al., 1991); flaccid tail—clinical severity score of 1; difficulty in righting after being flipped onto their backs—clinical severity score of 2; apparent hind limb weakness and not able to right themselves after being flipped onto their backs—clinical severity score of 3; severe hind limb weakness and walking upright only with difficulty—clinical severity score of 4; severe hind limb weakness and unable to upright—clinical severity score of 5; and hind limb paraplegia with no volitional leg movement—clinical severity score of 6. For some experimental groups the average cumulative disease score was determined. The cumulative disease score is the sum of the daily clinical score for an individual mouse over the observation period. The average cumulative disease score is the group's average daily clinical score summed over the observation period.

2.5. Treatment of actively-induced EAE

MAP peptides were prepared in saline at a concentration of 1.0 mg/ml. Peptide concentrations were adjusted by dilution with sterile saline as required just prior to treatment. Animals received the indicated amount of peptide (i.p.) in a volume of 0.2 ml.

2.6. T cell proliferation assay

Single cell suspensions of spleen cells were prepared from SJL mice previously immunized with PLP139–151/CFA. Cell proliferation responses to MAP peptides were assessed by plating 5×10^5 spleen cells in a 96-well tissue culture plate. The cell mixture was added in a 0.2 ml volume of stimulation media (RPMI 1640–5% fetal calf serum). The ACA derived MAP peptides, PLP 139–151 MAP peptide, or media control was added in a volume of 10 µl/well. Cultures were incubated for 72 h at 37 °C in 7% CO₂. Wells were pulsed for the final 18 h with 0.5 µCi per well [³H]thymidine (Amersham, Arlington Heights, IL). The cells were harvested onto glass fiber filters, and [³H]thymidine uptake was measured using a liquid scintillation counter (1205 Betaplate; Wallac, Turku, Finland). Mean cpm ±1 SD were calculated for quadruplicate wells.

We also used spleen cells from naive 5B6 transgenic mice that respond to the PLP 139–151 peptide. Cell proliferation responses to MAP peptides were assessed by plating 2×10^5 5B6 spleen cells/well in a 96-well tissue culture plate. The cell mixture was added in a 0.2 ml volume of stimulation media. The ACA derived MAP peptides, PLP 139–151 MAP peptide, or media control was added in a volume of 10 µl/well. The 5B6 spleen cells were cultured and harvested as described above.

2.7. Measurement of antibody response

Individual wells of a 96 well ELISA plate (NUNC Maxisorp) were coated with 0.1 ml (0.4 mM) of PLP 139–151 MAP, or ACA MAP derived

peptides and incubated overnight at 4 °C. All peptides used were diluted in a Carbonate Buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) at a final concentration of 0.4 mM. Plates were washed 4 times with PBS containing 0.5% Tween 20 (PBST). To prevent non-specific binding of immune serum to wells, plates were blocked for 2 h with PBS supplemented with 10% normal goat serum (0.2 ml/well). Wells were then washed with PBST. After washing immune serum from mice previously immunized with the PLP 139–151 peptide was added to individual wells (0.1 ml/well, diluted as indicated) and incubated at room temperature for 2 h. Plates were washed 4 times with 0.2 ml of PBST. Following washing 0.1 ml of horseradish peroxidase (HRP) conjugated Goat anti-mouse Ig (diluted as indicated in PBS) was added to individual wells and incubated for 1 h at room temperature. All wells were washed 4 times with 0.2 ml of PBST and then developed using 0.1 ml/well of the 3,3',5,5'-Tetramethylbenzidine (TMB) peroxidase substrate. After sufficient color development, 0.1 ml of 2 N sulfuric acid to stop the reaction was added to each well and absorbance was read at 450 nm.

2.8. Statistical analysis

Significant differences in the rate of EAE disease induction or the frequency of the development of relapse EAE were assessed using the log-rank method. A p value of 0.05 or less is considered significant. All values were calculated using GraphPad Prism Software, San Diego CA.

3. Results

3.1. A single amino acid change to the ACA 81–95 peptide enhances T cell recognition

The PLP 139–151 peptide and the ACA 81–95 peptide have identical amino acid residues at key MHC and TCR contact regions. The ACA 81–95 peptide is reported to be weakly encephalitogenic (Massilamany et al., 2010), a response that might be limited due to the influence of other amino acids within this peptide. As depicted in Table 1, the ACA 81–95 peptide has a leucine at amino acid position 86 while the PLP 139–151 encephalitogenic peptide has a glycine at this position. This position is adjacent to a TCR contact residue. In addition, the ACA peptide has asparagine at amino acid position 93 while the PLP 139–151 peptide has an aspartic acid at this position. Position 93 is adjacent to an amino acid that contributes to the MHC class II binding site. To determine the influence of these amino acids within the ACA peptide on recognition by T cells that proliferate in response to PLP 139–151 peptide, we synthesized the ACA peptide with a leucine to glycine substitution at ACA peptide position 86, keeping all other amino acids as found in the ACA peptide (Table 1). In addition we modified the ACA peptide at position 93 synthesizing a modified ACA peptide with a substitution of the asparagine with aspartic acid (ACA-93d). As is seen in Fig. 1, unlike

Table 1

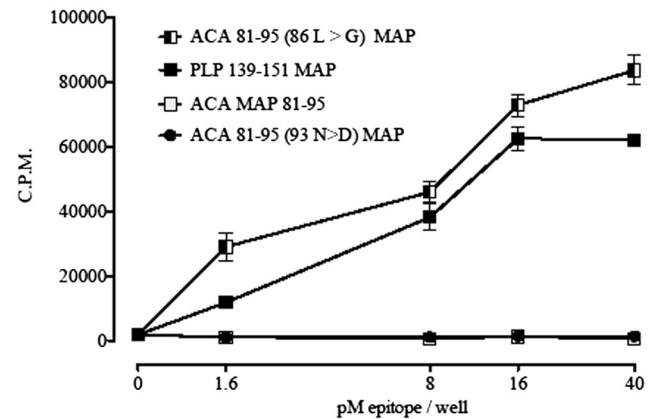
The amino acid sequence of the encephalitogenic peptide 139–151 from proteolipid protein and the peptide mimic derived from *Acanthamoeba castellanii* share MHC and TCR contact residues.

Peptide	Amino acid sequence ^a															
PLP 139–151				H	S	<u>L</u>	G	K	<u>W</u>	<u>L</u>	G	<u>H</u>	<u>P</u>	D	K	F
ACA 83–95				Y	F	L	L	K	W	L	G	H	P	N	V	S
ACA 81–95	R	G		Y	F	L	L	K	W	L	G	H	P	N	V	S
ACA 81–95 (86 L > G)	R	G		Y	F	L	<u>G</u>	K	W	L	G	H	P	N	V	S
ACA 81–95 (93 N > D)	R	G	Y	F	L	<u>L</u>	K	W	L	G	H		P	D	V	S

The peptide sequence 81–95 from the rhodanese-related sulfurtransferase of *Acanthamoeba castellanii* (ACA) shares the TCR and MHC class II binding residues with the encephalitogenic peptide sequence 139–151 of proteolipid protein (PLP). The ACA amino acids at positions 86 and 93 are distinct from the PLP 139–151 peptide sequence.

^a The amino acid sequence is shown for each peptide. For the PLP 139–151 peptide, the TCR binding amino acids are denoted by a rectangle and the MHC contact residues are bold and underlined. The amino acid substitutions for the ACA peptide are indicated as underlined.

A. Naive PLP TCR Transgenic



B. SJL PLP 139-151 Immune

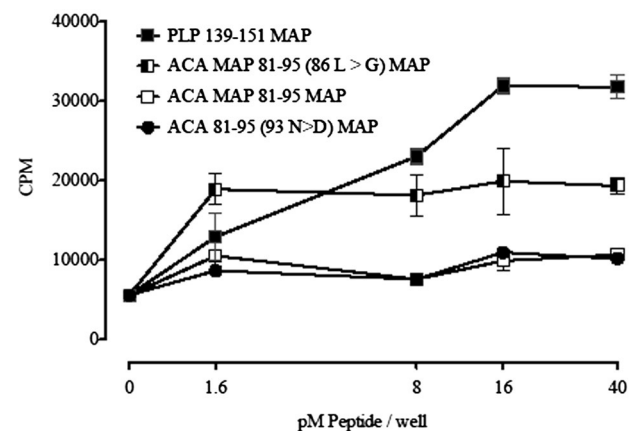


Fig. 1. T cell recognition of the nonlinear peptide octamers PLP_{139–151}MAP, ACA_{81–95}MAP, ACA_{86g}MAP and ACA_{93d}MAP. Single cell suspensions of spleen cells were prepared from 5B6 TCR transgenic SJL mice or from wild-type SJL mice previously immunized with PLP_{139–151}/CFA. Cell proliferation responses to MAP peptides were assessed by plating spleen cells in a 96-well tissue culture plate in the presence of MAP peptides with 3H-thymidine added the last day of the culture period. Plates were harvested and incorporated radioactivity determined as described in the methods section. A. Recognition by naive TCR transgenic T cells. B. Recognition by T cells from SJL mice previously immunized with the PLP 139–151 peptide.

the native ACA peptide, the leucine to glycine substitution allowed this modified ACA peptide (ACA-86g) to stimulate naive 5B6 transgenic T cells as well as lymphocytes from SJL mice immunized previously with the 139–151 encephalitogenic peptide. In contrast, the modification at ACA peptide position 93 with a change to aspartic acid did not enhance T cell recognition of the ACA peptide. We also found that the ACA-86g peptide was equally encephalitogenic in SJL mice when compared to the PLP 139–151 peptide (Table 2). Table 2 also shows that the ACA-86g peptide was similar to the PLP 139–151 peptide in stimulating the production of IL-17 in the presence of spleen cells from SJL mice immunized previously with the PLP 139–151 encephalitogenic peptide. The ACA-93d peptide did not provide additional stimulation for the production of IL-17 compared to the original ACA 81–95 sequence.

3.2. The ACA 81–95 peptide escapes antibody recognition

To determine if the ACA-86g modification changed the humoral recognition of the ACA peptide we used antisera from mice previously immunized with the 139–151 encephalitogenic peptide. As seen in Table 3, neither the ACA peptide nor the ACA-86g peptide is recognized by sera from mice recovered from PLP 139–151 induced EAE, sera that

Table 2

A single amino acid change enhances the T cell recognition and encephalitogenicity of the ACA 81–95 peptide.

A.	Peptides used to stimulate PLP 139–151 immune spleen cells			
	PLP 139–151 MAP	ACA 81–95 86G MAP	ACA 81–95 93D MAP	ACA 81–95 MAP
IL-17a Production (pg/ml)	6926 $\pm$ 250	8933 $\pm$ 1640	2578 $\pm$ 495 [#]	2761 $\pm$ 123 [#]
IL-10 Production (pg/ml)	199 $\pm$ 52	387 $\pm$ 76 [@]	101 $\pm$ 63	38 $\pm$ 15 [#]
B.	Encephalitogens used to induce EAE in naive SJL mice			
	PLP 139–151/CFA	ACA 81–95 86G/CFA	ACA 81–95 93D/CFA	ACA 81–95/CFA
Percent with EAE	87% (20/23)	82% (23/28)	N.D.	50% (2/4)
Day of EAE onset	14.4 $\pm$ 2.8	14.7 $\pm$ 5.6		12.5 $\pm$ 0.7
Disease severity	5.7 $\pm$ 1.7	4.7 $\pm$ 2.1		2.0 $\pm$ 1.4

A. PLP 139–151 immune SJL spleen cells were cultured at 4 million cells per ml. Spleen cells were stimulated with equal molar concentrations of the various MAP peptides (4 μ g/ml for PLP 139–151 MAP). After 72 h of in vitro culture supernatants were collected for analysis of cytokine production. PLP 139–151 immune spleen cells produced high levels of IL-17a in response to PLP 139–151 MAP and ACA 86G MAP. Levels of IL-17a production in response to ACA 93D MAP and ACA MAP stimulation were significantly less than for the control peptide PLP 139–151. IL-10 cytokine production was significantly higher for the ACA 86G peptide than for the control PLP 139–151 MAP peptide. p value of less than 0.05 indicated by @, p value less than 0.005 indicated by #.

B. To determine the encephalitogenicity of the modified ACA peptide, naive SJL mice were challenged with ACA derived peptides emulsified in CFA.

detects the PLP 139–151 peptide used for immunization. We explored additional modifications to the ACA 81–95 peptide in order to evaluate in greater detail the impact of specific amino acids contained in the native ACA 81–95 sequence on recognition by T cells specific to the PLP 139–151 encephalitogenic peptide or by antibody obtained from SJL mice previously immunized with the PLP 139–151 peptide. Changing the amino acid at ACA position 93 from an asparagine to aspartic does not enhance recognition of the ACA-93d peptide by PLP 139–151 peptide specific T cells. The ACA-93d substitution in combination with the ACA-86g substitution does not improve the T cell response beyond that is seen with the ACA-86g single substituted peptide. However in all cases these changes to the ACA sequence do not lead to humoral recognition of these three peptides by antiserum that reacts with the PLP 139–151 peptide.

3.3. Aromatic amino acids block antibody recognition

The ACA 81–95 peptide is not recognized by immune serum from PLP 139–151 immunized mice. By deleting single amino acids from the ACA 81–95 peptide sequence we found that the amino acids at positions 83 and 84 of the ACA peptide contribute to this escape from recognition by PLP 139–151 peptide specific immune serum. As shown in Table 3, deletion of tyrosine from position 83 and phenylalanine from position 84 now allows humoral detection of this modified ACA 81–95 peptide by antiserum from mice immunized with PLP 139–151 peptide. The T cell recognition of the ACA peptide deleted for these amino acids at positions 83 and 84 is not changed and demonstrates the influence of position 86 for T cell recognition and of position 84 for antibody recognition of modified ACA peptides using T cells or

Table 3The contribution of amino acids within the 81–95 peptide derived from *Acanthamoeba castellanii* to recognition by the humoral and cellular response specific to the 139–151 encephalitogenic peptide from proteolipid protein.

Peptide designation	Amino acid sequence	Immune serum ^a	Transgenic T cells ^b
		O.D.	S.I.
PLP 139–151 MAP ^c (HSLGKWLGHDPKFGG) ₈ -K ₄ -K ₂ -K- β A		0.65	22
5K + PLP 139–151 MAP	(KKKKKHSLSGKWLGHDPKFGG) ₈ -K ₄ -K ₂ -K- β A	0.31	19
PLP 141–149 MAP	(LGKWLGHDPKFGG) ₈ -K ₄ -K ₂ -K- β A	0.42	6
ACA 81–95 MAP ^d	(KRGYFLKWLGHDPNVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.05	2
ACA 81–95 MAP (93 N > D)	(KRGYFLKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.05	2
ACA 81–95 MAP (86 L > G)	(KRGYFLGKWLGHDPNVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.05	21
ACA 81–95 MAP (86 L > A)	(KRGYFLAKWLGHDPNVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.05	17
ACA 81–95 MAP (86 L > G, 93 N > D)	(KRGYFLGKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.05	20
ACA 81–95 MAP (86 L > G, 93 N > D, –84F)	(KRGY- <u>L</u> GKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.37	17
ACA 81–95 MAP (86 L > G, –85Y)	(KRG- <u>FL</u> GKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.15	18
ACA 81–95 MAP (86 L > G, 93 N > D, –83Y–84F)	(KRG- <u>LG</u> KWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.46	16
ACA 83–95 MAP (86 L > G, 93 N > D)	(YFLGKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.14	19
ACA 84–95 MAP (86 L > G, 93 N > D)	(FLGKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.17	15
ACA 85–95 MAP (86 L > G, 93 N > D)	(LGKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.41	11
K + ACA 85–95 MAP (86 L > G, 93 N > D)	(KLKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.28	9
2K + ACA 85–95 MAP (86 L > G, 93 N > D)	(KKLGKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.13	18
3K + ACA 85–95 MAP (86 L > G, 93 N > D)	(KKKLGKWLGHDPVSKGG) ₈ -K ₄ -K ₂ -K- β A	0.14	16

The contribution of the peptide sequence to humoral and T cell recognition of PLP and ACA derived peptides. T cell recognition of the ACA peptide by PLP 139–151 peptide specific T cells requires modification at amino acid position 86. Recognition of the ACA peptide by antiserum from SJL mice immunized with PLP 139–151 is prevented by ACA peptides at positions 83 and 84.

^a Immune serum from PLP 139–151 immunized animals was collected as described in Materials and methods. Reactivity of the peptides listed against PLP 139–151 immune serum was assessed by ELISA as described in Materials and methods and the relative strength of the response shown, reported in O.D.

^b A proliferative assay was used to assess the capacity of the peptides listed to stimulate TCR transgenic PLP 139–151 specific T cells as described in Materials and methods. The relative strength of the proliferative response is shown as a comparison to the control PLP 139–151 peptide response and is reported as the stimulation index (S.I.) experimental/control.

^c At amino acid position 140 the native cysteine has been replaced with a serine. A spacer consisting of two glycines was added to separate the PLP peptide from the branched lysine scaffold.

^d To improve solubility of the ACA 81–95 derived peptide MAPs lysine residues were added to the N-termini and also to the di-glycine spacer.

antiserum from PLP 139–151 peptide immunized mice. Peptides synthesized with lysine at position 83 and 84 reduce antibody recognition but do not impact antibody recognition to the degree provided by the native amino acids, tyrosine and phenylalanine. Thus the ACA peptide changed at position 86 from a leucine to glycine allows recognition of this modified ACA peptide by PLP 139–151 peptide specific T cells. The presence of the phenylalanine and tyrosine at positions 83 and 84 provide the natural modification to the ACA peptide such that it is not recognized by antiserum from mice immunized with the PLP 139–151 encephalitogenic peptide.

3.4. The ACA_{81–95}MAP peptide has a limited impact on rEAE

The observation that the ACA peptide was not recognized by antiserum from PLP 139–151 immunized animals and yet stimulated PLP-139-151 specific T cells suggested that anaphylaxis may not be an issue if the ACA peptide was used as a therapeutic to modify the development of the relapsing phase of EAE. Our initial experiments used non-linear peptide octamers of the ACA peptide to prevent rEAE in SJL mice immunized previously with the PLP 139–151 peptide. As shown in Fig. 2, we treated mice with the ACA peptide-octamer after the mice had recovered from primary disease induced by immunization with the PLP 139–151 peptide. Treatment was initiated one day after all signs of primary disease were no longer evident and treatment with the ACA peptide-octamer was initiated as an intraperitoneal injection of 100 µg of the reagent in saline and continued for 7 consecutive days. The treatment with the ACA peptide-octamer prevented or delayed the development of the relapsing stage of EAE in some of the treated mice, however the treatment effect, while suggestive, did not reach statistical significance. Importantly, none of the mice treated with the ACA peptide-octamer showed signs of anaphylaxis during the treatment with this reagent in soluble form.

3.5. The modified ACA-86gMAP peptide prevents rEAE

The ACA 81–95 peptide with the substitution of a glycine at position 86 is encephalitogenic and recognized by T cells from SJL mice previously immunized with the PLP 139–151 encephalitogenic peptide. We tested the ACA-86g peptide octamer for its influence on the development of the relapsing phase of disease. SJL mice immunized with the PLP 139–151 encephalitogenic peptide were treated with the ACA-86g peptide octamer after the mice had recovered from the primary episode of

disease. Treatment with the ACA-86g peptide octamer prevented all signs of rEAE for 70 days in 12/14 mice as which point in the experiments 2/14 mice developed minimal signs of clinical disease from which they quickly recovered (Fig. 3). Fig. 3 also shows that treatment of PLP 139–151 peptide immunized mice with an octamer peptide of PLP 178–191 was without effect in preventing the relapsing stage of disease and equivalent to the saline vehicle control group. Thus treatment with the ACA-86g peptide was effective as a therapeutic agent in preventing the relapsing stage of EAE in PLP 139–151 peptide immunized SJL mice, a therapy that was not achieved with the PLP 178–191 peptide octamer. No signs of anaphylaxis were evident in any mice treated with the ACA-86g MAP peptide during the successful treatment for prevention of the relapsing stage of EAE in SJL mice immunized with the PLP 139–151 encephalitogenic peptide.

4. Discussion

Antigen specific peptides are used to induce epitope specific tolerance and to cause some deviation of a developing autoimmune response (Elliott et al., 1996; Devaux et al., 1997; Karin et al., 1998; Leadbetter et al., 1998; Kobayashi et al., 2008). In general these observations support the use of peptide-specific therapy as a means to modify T-cell dependent autoimmune disease. While there is much evidence supporting this approach to prevent the development of autoimmune

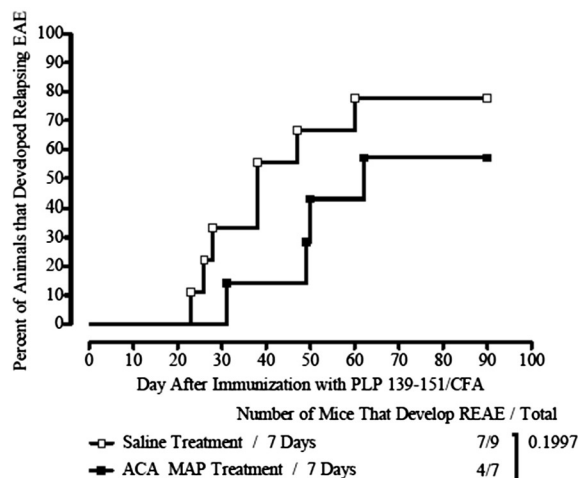


Fig. 2. The development of relapsing EAE in SJL mice previously immunized with the PLP 139–151 encephalitogenic peptide. Following recovery from primary disease mice were treated with the ACA_{81–95}MAP reagent. Mice were administered the MAP reagent or control for 7 consecutive days with treatment started when all signs of the primary clinical disease were resolved. All injections were by the intraperitoneal route, 100 µg/injection.

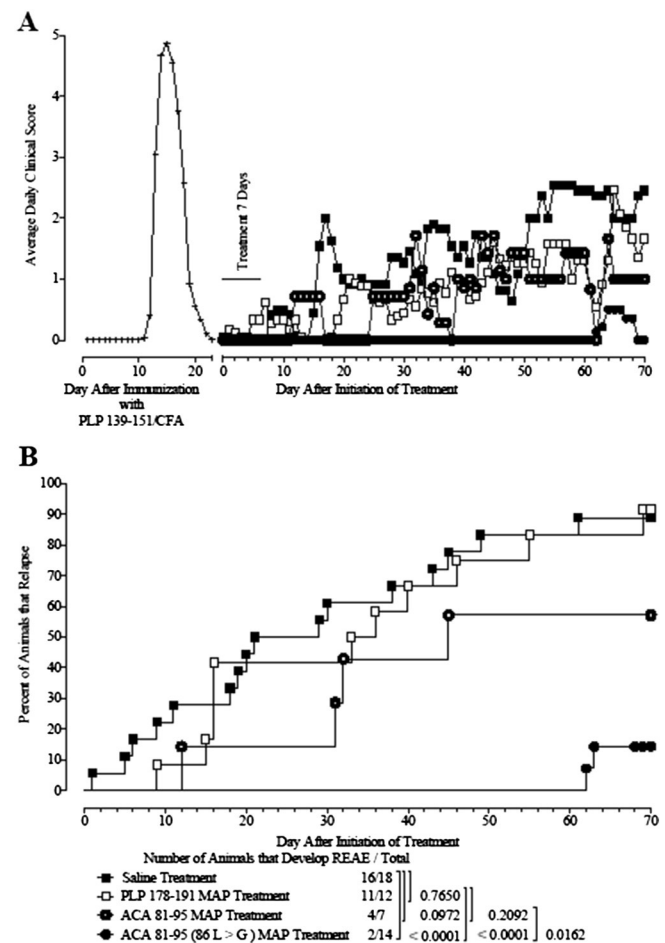


Fig. 3. Prevention of relapsing EAE in SJL mice previously immunized with the PLP 139–151 encephalitogenic peptide. Following recovery from primary disease mice were treated with the ACA_{81–95}MAP or PLP_{178–191}MAP reagents. Mice were administered the MAP reagents or control for 7 consecutive days with treatment started when all signs of the primary clinical disease were resolved. All injections were by the intraperitoneal route, 100 µg/injection. A. Average clinical score. B. Percentage of mice that developed relapses following treatment.

disease, often when this therapy is used to treat clinically apparent disease a humoral response to the therapeutic peptide becomes evident, often leading to a peptide-specific anaphylactic response (Pedotti et al., 2001; Pedotti et al., 2003; C.E. Smith et al., 2005; Lichtenegger et al., 2007). Consequently for full utility of peptide-mediated therapy, strategies are required to evade the antibody-mediated negative outcome that develops frequently while retaining the immunoregulation provided by the specific peptide.

Following immunization with the PLP 139–151 peptide SJL mice develop of rEAE. This response is also accompanied by a humoral response to the peptide used for immunization that results in the development of an anaphylactic response when this peptide is administered in soluble form following the initial immunization, a result that prevents the therapeutic administration of this peptide in soluble form (Wegmann et al., 2008). In this study we have found that the PLP 139–151 peptide mimic derived from ACA is not recognized by antibodies from SJL mice immunized with the PLP 139–151 peptide (Table 3). Although the ACA peptide contains the MHC and TCR contact residues found for PLP 139–151 the ACA peptide has diminished encephalitogenicity (Massilamany et al., 2010), and is not well-recognized by T cells from PLP 139–151 immunized SJL mice (Fig. 1, Table 3). We found that changing the amino acid at position 86 of the ACA peptide from a leucine to a glycine resulted in a significant increase in encephalitogenicity as well as its recognition by T cells derived from mice previously immunized with the PLP 139–151 encephalitogenic peptide (Fig. 1, Table 2). Although this L > G modification resulted in significantly improved recognition by PLP 139–151 encephalitogenic T cells, this modification did not result in humoral recognition by antibodies derived from PLP 139–151 immunized mice (Table 3).

Since the modified ACA-86g peptide was not recognized by antibody from PLP 139–151 immunized mice we administered this peptide to SJL mice that had recovered from the first episode of EAE induced by immunization with the PLP 139–151 encephalitogen. Mice treated with the ACA-86g peptide did not develop rEAE whereas mice treated with PLP_{178–191}MAP or with the diluent control all developed rEAE as depicted in Fig. 3. This inhibition of rEAE by using the ACA-86g peptide that is recognized by T cells elicited by immunization with PLP 139–151 suggests that PLP 139–151 specific T cells have the potential to contribute to the relapsing phase of disease and that this contribution is thwarted by treatment with the ACA-86g MAP peptide. The ACA-86g MAP peptide did not influence the development of rEAE in SJL mice in which primary disease was induced by immunization with the PLP 178–191 encephalitogenic peptide (data not shown).

We have shown previously that targeting T cells with MAP peptides containing the priming encephalitogen prevented the development of rEAE when the MAP peptides were administered following recovery from primary disease (Wegmann et al., 2013). We have used MAP peptides for these and previous studies because of the improved stability that the MAP peptide configuration provides following administration in vivo (Bracci et al., 2003). The encephalitogen we used in this recent study, the oligodendrocyte-specific protein (OSP)/claudin-11, has been reported to elicit focused T cell responses following immunization with this peptide (Kaushansky et al., 2008). However, relapsing EAE develops in SJL mice following immunization with the encephalitogenic OSP 55–71 peptide and our success in preventing rEAE with the OSP_{55–71}MAP peptide may reflect the focused T cell response as reported for this encephalitogen.

It should be pointed out that the autoimmune response initiated following immunization of SJL mice with the PLP 139–151 encephalitogenic peptide has been described as one that allows the development of T cell responses to additional myelin-derived epitopes, responses that are associated with the relapsing episodes of disease (Lehmann et al., 1992; McRae et al., 1995; McMahon et al., 2005; Bailey et al., 2007). These studies assume that the relapsing phase of disease is a consequence of the pathogenic relevance of the T cell populations that develop as a consequence of the initial disease process. Thus, successful

prevention of the relapsing phase of EAE with a MAP peptide that targets T cells stimulated by the immunizing PLP 139–151 peptide is somewhat unexpected but in keeping with other reports concerning the specificity of the encephalitogenic response at the relapsing phase of EAE (Kroenke and Segal, 2007). In addition, these results are essentially identical to our findings using the OSP 55–71 peptide as the priming encephalitogenic peptide in which rEAE was prevented by treatment with OSP_{55–71}MAP, a reagent that targets T cells stimulated by the priming immunization (Wegmann et al., 2013).

Whether immunoregulatory T cells reactive with PLP 139–151 are stimulated by treatment with the ACA-86g MAP and subsequently prevent the development of rEAE is one possible explanation for these results shown in this study. It may also be that the MAP peptides cause peptide-specific T cells to alter their trafficking pattern from the target tissue to any cell that has processed the MAP peptide in the context of MHC class II molecules. We have previously reported that treatment with MAP peptides cause a peptide specific accumulation of the T cells in the spleen and significantly reduce this population in the CNS (Wegmann et al., 2008).

The various immunologic contributions that result in the expression of the relapses seen in mouse models of EAE remain unresolved. Many studies provide evidence for a patterned spread to pathogenic epitopes by T cells apparently stimulated during the primary disease process (Lehmann et al., 1992; Karandikar et al., 2000; McMahon et al., 2005; Bailey et al., 2007; Getts et al., 2012) and describe inhibition of the relapsing phase of the disease by targeting peptides identified as part of the spreading cascade. There are also many studies that question the role of pathogenic epitope spread in relapsing models of EAE and provide evidence suggesting that the epitope that is the target of T cells causing the primary disease is responsible for rEAE as the target of T cell causing the relapsing phase of disease (Su and Sriram, 1991; P.A. Smith et al., 2005; Kroenke and Segal, 2007; Li et al., 2011; Wegmann et al., 2013). It is important to establish the basis for relapsing disease in autoimmune models that exhibit a relapsing/remitting course of disease in that peptide specific strategies for clinical intervention of autoimmunity are guided by results from relevant animal models. As the eliciting peptides are identified our findings with MAP peptides, as seen in our studies with the EAE model, may also have clinical relevance.

The results from our study are consistent with a single T cell specificity as responsible for rEAE in the SJL mouse model, a T cell population that has its origin from the primary disease episode. By modifying the ACA peptide sequence we have been able to develop a peptide that is recognized by T cells initially stimulated with the PLP 139–151 peptide immunogen but not recognized by antiserum from these same immunized mice. This has allowed us to treat SJL mice that have recovered from primary EAE induced by the PLP 139–151 encephalitogenic peptide with the modified ACA peptide (ACA-86g). Administration of this peptide to mice after recovery from primary disease prevents rEAE in all mice treated with this peptide and occurs without any signs of anaphylaxis. Peptide-specific protection from relapsing autoimmune disease may depend on knowledge of the priming epitope that elicits the initial disease rather than a search for secondary targets. The effectiveness of MAP peptides as reagents for clinical use should be considered as peptide-specific treatment strategies continue to evolve.

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