Liposome-mediated peptide loading of MHC-DR molecules in vivo

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Abstract Amino acid residues 3–15 of mycobacterial HSP60 define a dominant T-cell epitope for *HLA-DR3+ve* humans and *Mamu-DR3+ve* rhesus monkeys. Our results show that *Mamu-DR3* molecules on PBMC can be efficiently loaded in vivo with the above-mentioned peptides when they are intravenously injected encapsulated in liposomes, but not in the free form. Mamu-DR3 loading is abolished by encapsulation of a nonstimulatory peptide. These results have implications for the delivery of therapeutic peptides in vivo.

Key words: (Rhesus monkey); MHC; Antigen presentation; Immunotherapy

1. Introduction

T-cell recognition of antigens which are presented in the context of MHC class II molecules is a key-process in normal and pathological processes of the immune system [1,2]. Interference in the recognition process with competitor peptides (CP) such as nonstimulatory analogues of T-cell stimulatory peptides (SP) is regarded as a specific approach for therapy of autoimmune diseases [3]. Indeed promising results have been obtained in disease models, such as autoimmune encephalomyelitis, in inbred strains of mice and rats [4,5]. However, data in outbred species such as nonhuman primates, which are phylogenetically closely related to humans, are scarce.

In the present study we have investigated the in vivo loading of rhesus monkey *Mamu-DR3* molecules with peptides derived from the 65 kDa heat-shock protein (HSP65) of *Mycobacterium tuberculosis*. The overlapping peptides HSPp3-13 and HSPp4-15 were used as SP since they contain an immunodominant T-cell epitope in *HLA-DR3* positive individuals [6,7] and also for the evolutionary equivalent molecules in rhesus monkeys, named *Mamu-DR3* [8]. A nonstimulatory variant peptide of p4-15 (p4-15 E→R), which binds with the same kinetics to *Mamu-DR3* as SP and therefore prevents the loading of SP and subsequent T-cell stimulation in vitro [9], was selected as CP.

The SP-*Mamu-DR3* complex is recognized by several SP-specific and *HLA-DR3*-restricted human T-cell clones [8]. This phenomenon of trans-species presentation of antigen to human T cells has been demonstrated also with APC from other nonhuman primate species in combination with various antigens [10–12]. This principle enabled us to monitor the in vivo loading of *Mamu-DR3* molecules in rhesus monkeys by proliferation-induction of the cross-reactive *HLA-DR3* restricted human T-cell clones.

The aim of this study was to investigate the possibility of in vivo loading of *Mamu-DR3* molecules with CP. Because CP does not stimulate the T-cell clones, we have first investigated loading of SP and then tested if loading of SP could be prevented with CP.

Our results show that after bolus injection of SP in the free form to a dose of 25 mg free SP is detectable in venous blood plasma but disappears rapidly from the circulation. PBMC isolated from the same blood samples failed to activate the T-cell clones indicating that loading of *Mamu-DR3* molecules with the SP at significant level had not taken place. A similar observation has been reported by Grey and co-workers, namely that in vivo administered peptides fail to load murine I-A^b molecule [13].

Similar experiments were performed with SP encapsulated in unselected liposomes. In this situation only low plasma levels of SP were found but PBMC isolated from the same blood samples displayed a high stimulatory capacity of the human T-cell clones. Even after injection of only 300 μg of SP encapsulated in the liposomes significant stimulatory capacity of the PBMC was found. PBMC isolated after injection of liposomes which contained besides SP also CP, were unable to induce proliferation of the clones, indicating that loading of *Mamu-DR3* molecules was abolished. These results show that rhesus monkeys can be used to investigate the conditions for in vivo loading of MHC class II molecules on APC.

2. Materials and methods

2.1. Animals

Each animal in the pedigreed rhesus monkey colony at the Biomedical Primate Research Centre, which is located in Rijswijk, The Netherlands, has been typed for most known MHC class I and II molecules. For inclusion in the present study PBMC from a panel of *Mamu-DR3*-positive monkeys was tested for the capacity to present the SP to a set of p3-13-specific and *HLA-DR3*-restricted T-cell clones [7,8]. Two high responder monkeys (1RA, female, birth August 1983; and 4051, male, birth December 1988) were selected on these criteria. Both monkeys lacked antibodies and a proliferative T-cell response to the SP.

The experiments reported here have been approved by the Institute's Animal Care and User Committee.

2.2. Antigens

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer at 48×10 μmol scale (Abimed AMS 422) [14] using TentagelS AC as a resin (Rapp, Tübingen, Germany) [15].

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Abbreviations: HSP, heat-shock protein; CP, competitor peptide; MHC, major histocompatibility complex (*HLA* in man; *Mamu* in rhesus monkey); PBMC, peripheral blood mononuclear cell(s); SP, stimulatory peptide

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Repetitive couplings were performed by adding a mixture of 90 μl of 0.67 M PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate [14] in NMP (*N*-methylpyrrolidone), 20 μl of NMM (*N*-methylmorpholine) in NMP 2/1 (v/v) and 100 μl of a 0.60 M solution of the appropriate Fmoc (9-fluorenylmethoxycarbonyl) amino acids [16,17] in NMP (6-fold excess) to each reaction vessel. At 70% of the reaction time approximately 50 μl of dichloromethane was added to each reaction vessel. Fmoc-deprotection was performed by adding 3 times 0.8 ml of piperidine/DMA (*N*,*N*-dimethylacetamide) 1/4 (v/v) to each reaction vessel. Coupling and deprotection times were increased as the synthesis proceeded, starting with 60 min and 3 times 3 min, respectively. Washing after coupling and Fmoc deprotection steps were done with 6 times 1.2 ml of DMA. Cleavage of the peptides was performed with TFA (trifluoroacetic acid)/water 19/1 (v/v) for 3 h. Peptides were collected by ether precipitation, dissolved in water, centrifugated and lyophilized. Quantitation was done by OD measurement at 276 nm using $\varepsilon_y = 1400$. The purity of the peptides was determined by analytical reversed phase HPLC using a water–acetonitrile gradient containing 0.1% TFA and proved to be always greater than 90% (UV at 214 nm). The integrity of the peptides was determined by laser-desorption time-of-flight mass spectrometry (TOF-MALDI) on a Lasermat mass spectrometer (Finnigan MAT, UK) using ACH (α-cyano-4-hydroxycinnamic acid; 10 mg/ml in acetonitrile/water 60/40 v/v) containing 0.1% as a matrix. Calibration was performed with a peptide of known molecular mass as internal reference.

Three peptides were synthesized: (i) p3-13 (KTIAYDEEARR); (ii) protected p4-15 (TIAYDEEARRGL-NH_2) and (iii) a p4-15 analogue which fails to activate the T-cell clones used in this study as consequence of a E→R substitution at position 9 (TIAYDREARRGL-NH_2), which is one of three TCR contacting residues. All three peptides bind to *HLA-* and *Mamu-DR3* molecules due to the presence of the motif -I(AY)D- [8]. To date, identical results have been obtained when wither p3-13 or p4-15 were used as SP of the human clones. Prepulse experiments have shown that the nonstimulatory p4-15 analogue interferes only with binding of p4-15 to the *Mamu-DR3* molecule but not with binding to the TCR.

2.3. Liposome preparation

Partially hydrogenated egg-phosphatidylcholine with an iodine value of 40 (PHEPC) was purchased from Asahi Chemical Industry Co. Ltd. (Ibarakiken, Japan). Egg-phosphatidylcholesterol (EPG) was obtained from Lipoid (Ludwigshafen, Germany). Cholesterol (Chol) was from Sigma Chem. Co. (St. Louis, MO). A lipid mixture of PHEPC/EPG/Chol (10:1:4 molar ratio) was prepared in chloroform/methanol (10:1) and dried to a thin film by rotary evaporation. After evacuation for at least 1 h the lipid film was hydrated in HEPES buffer (50 mM HEPES, 73 mM NaCl, pH 8.0; 1 mmol phospholipid/ml) containing effector peptide (1.6 mg/ml) and/or competitor peptide (62.5 mg/ml). Nonentrapped peptide was removed by ultracentifugation at 160 000×*g* during 30 min. After decantation of the supernatant the pellet was resuspended and subsequently washed 4 times with HEPES buffer (10 mM HEPES, 140 mM NaCl, pH 7.4). The dispersions were diluted with 10 mM HEPES buffer to a final phospholipid concentration of 0.2 mmol/ml. The final peptide-to-phospholipid ratio of the dispersions amounted to 1.1 mg/mmol in case of SP liposome formulations, 23.7 mg/mmol in case of the CP liposome formulation and 0.54 and 21.3 mg/mmol, respectively, for the liposomes containing both effector and competitor peptide. Liposome dispersions were stored at 4°C under nitrogen atmosphere for maximally 1 week.

2.4. Test substance administration and blood sampling

Test substance administration and blood sampling were performed in fully conscious monkeys, which were placed in a restraint chair. The method was chosen because repeated sedation could avoided. When appropriately trained before starting the experiment, this procedure causes only negligible inconvenience to the monkeys. Test substances were injected as bolus into the vena saphena and blood samples were collected in heparinized vacutainer tubes from the vena saphena in the contralateral leg.

2.5. Experimental design

Just before and at different time points after test-substance administration venous blood samples were collected. Plasma and blood cells were separated by centrifugation for 10 min at 200×*g*. After collecting plasma, the pelleted cells were resuspended in PBS and PBMC were isolated by density gradient centrifugation on Lymphocyte Separation Medium (LSM: Organon-Technika Corp., Durham, NC) for 25 min at 280×*g*. Isolated PBMC were washed twice with PBS, resuspended in RPMI containing 10% pooled human serum and kept stored at 4°C until testing for proliferation induction of T-cell clones.

2.6. T-cell clones

Three p3-13 specific and *HLA-DR3* restricted human T-cell clones (CAA p151-1, DAA p151-1 and DAA p151-2) were used [7]. Proliferation was assayed by mixing 10^4 T cells with 5×10^4 irradiated PBMC (50 Gy) as APC in 96-well flatbottom microtiter plates in triplicate. After 66 h of culture, 0.5 μCi [^{3}H]thymidine (NEN Dupont; 6.7 mCi/ml specific activity) was added to each well and 18 h later cells were collected on glass filters. To determine incorporation of radiolabel, DNA was precipitated on glass filters and ^{3}H levels were measured by liquid scintillation counting.

2.7. Measurement of SP levels in plasma

T-cell clones were cultured with irradiated PBMC from a *HLA-DR3*-matched human donor as APC. Rhesus monkey plasma samples were added to the cultures as antigen source to a final concentration of 10% (v/v). In parallel cultures logarithmic dilutions of SP were added. Proliferation was determined as described above.

2.8. Measurement of SP and CP presentation by PBMC

PBMC were isolated from the rhesus monkey venous blood samples, irradiated at 50 Gy and cultured with the three T-cell clones. Proliferation was measured as described above.

CP loading in the *Mamu-DR3* molecules was measured indirectly namely by inhibition of SP presentation, as described above. For effective blocking of SP presentation in culture 100-fold excess of CP is needed [9]. Because the maximal amount of peptide that could be entrapped in the administered volume of liposomes (5 ml) proved to be 30 mg the following liposomes containing 30 mg CP, 0.3 mg SP or 30 mg CP +0.3 mg SP were prepared.

2.9. Normalization of the results

The substantial day-to-day variation of the proliferative response of the T-cell clones to the SP under standard conditions necessitated normalization of results. For normalization, cpm measured in parallel cultures of the clones stimulated with 1 μg SP and human APC were set at 100%. The cpm from cultures belonging to the same series are expressed as percentage of this control value.

3. Results and discussion

3.1. Intravenous injection of free SP

To determine whether intravenously injected SP binds to *Mamu-DR3* molecules in vivo, a rhesus monkeys were injected with three consecutive doses of 1, 5 and 25 mg SP at 1 h interval. Venous blood samples were collected at 2, 5, 10 and 60 min after each injection and the presence of SP in plasma or bound to PBMC was assayed by stimulation of the human T-cell clones. As shown by the typical example depicted in Fig. 1, no significant stimulatory activity of PBMC was found. Analysis of the plasma fraction of the blood samples using human APC shows presence of SP, which remained detectable for at least 10 min after injection. It is therefore concluded that effective loading of *MHC-DR* molecules on PBMC with intravenously injected SP in the free form did not take place.

In a 3 day culture of rhesus monkey PBMC with the human T-cell clones a SP concentration of less than 1 μg/ml is sufficient to induce loading of *Mamu-DR3* molecules on the PBMC as deduced from proliferation of the T cells. A SP dose of 25 mg given to a monkey of 5 kg with a blood volume of about 300 ml results in a theoretical plasma concentration of about 75 μg/ml. Consequently absence of *Mamu-DR3* loading with injected free SP is likely not due to insufficient plas-

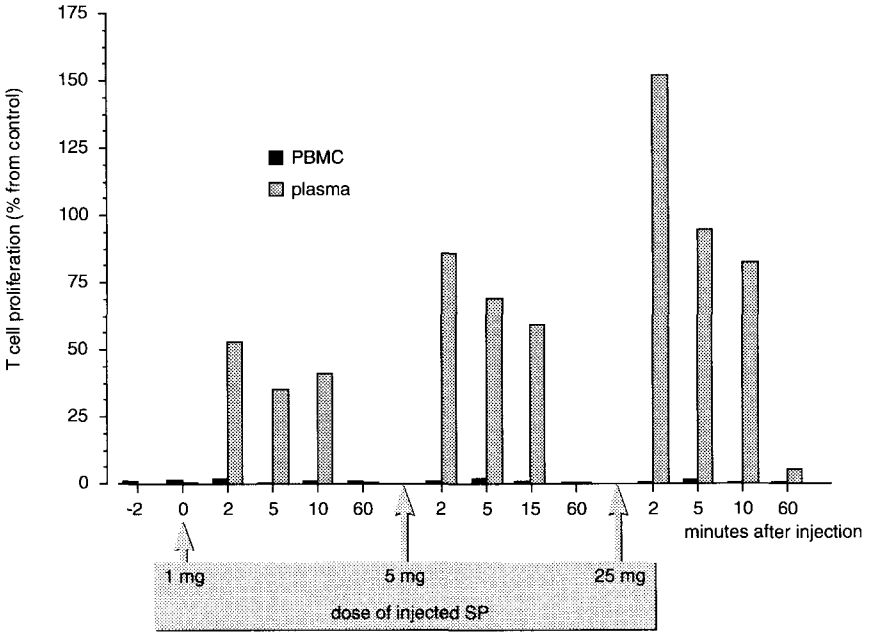
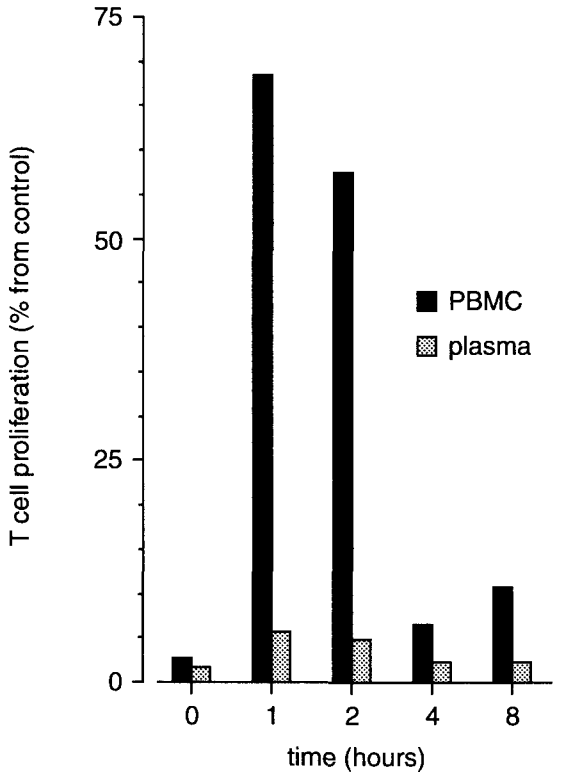


Fig. 1. SP levels in plasma and bound to PBMC when injected as free peptide. Rhesus monkey 1RA was injected with three consecutive doses of free SP: 1 mg ($t=0$), 5 mg ($t=75$ min) and 25 mg ($t=150$ min). At the indicated time points venous blood samples were collected and plasma and PBMC were separated by centrifugation. The SP levels in plasma (white bars) were determined by proliferation–induction of the T-cell clones with human APC and 10% plasma (open bars) as antigen source. Cell-bound SP (closed bars) was determined by culturing the T-cell clones with irradiated monkey PBMC. Proliferation assays were performed as described in Section 2. The results obtained with T-cell clone CAAp151-1 are shown as representative example. Normalization of the results was performed as described in Section 2.

ma levels. A more plausible explanation is that pharmacokinetic properties of the peptide are too poor for efficient binding to the relevant MHC class II molecules. A similar conclusion has been reached based on experiments in mice [13].



3.2. *Intravenous injection of liposome-encapsulated SP*

Encapsulation of SP into liposomes may prolong the blood circulation time of the peptide and enhance uptake by blood monocytes [18,19]. Importantly, ingested particles arrive in the phagolysomes, being a cell compartment where degradation of the particles and loading of MHC class II molecules may take place [25]. To investigate this possibility, two monkeys were intravenously injected with a single liposome dose containing 2.5 mg SP. At 1, 2, 4 and 8 h after injection, plasma and PBMC were collected and again assayed for the presentation of SP by proliferation–induction of the T cell clones. The results in Fig. 2 show that PBMC collected at 1 and 2 h after injection of liposome-encapsulated SP stimulate proliferation of the T-cell clones, whereas only negligible stimulatory activity was found in the plasma. This experiment clearly demonstrates that liposome-encapsulation of SP facilitates loading of *Mamu-DR* molecules on PBMC.

In a next experiment the dose–effect relationship and the time course of *Mamu-DR3* loading with SP were further investigated. One monkey was injected at 3 h intervals with liposomes containing 0.3, 1.0 and 3.0 mg SP, respectively.

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Fig. 2. SP levels in plasma and bound to PBMC upon injection of SP containing liposomes. Rhesus monkey 4051 was injected with 5 ml of liposome suspension containing 2.5 mg SP. At 1, 2, 4 and 8 h after injection venous blood samples of 2.5 ml each were collected. Plasma and cells were separated by centrifugation. Loading of PBMC with SP (closed bars) or SP activity in plasma (open bars) was assayed. The SP levels in plasma were determined by proliferation–induction of the T-cell clones with human APC and 10% plasma as antigen source. Cell-bound SP (closed bars) was determined by culturing the T-cell clones with irradiated monkey PBMC. The results obtained with monkey 4051 and T-cell clone CAAp151-1 as read-out system are shown as a representative example. Normalization of the results was performed as described in Section 2.

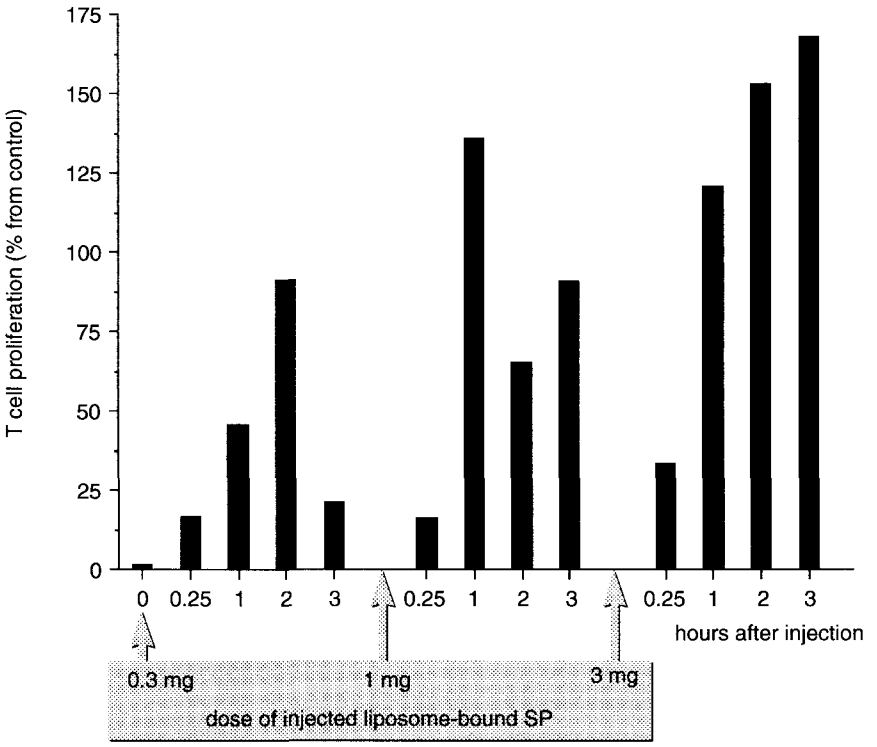

Fig. 3. Dose–response relationship of i.v. injected liposome-encapsulated SP. Rhesus monkey 4051 was injected intravenously with three doses of SP-containing liposomes. At $t=0$ h liposomes containing 0.3 mg SP were injected, followed by liposomes containing 1.0 mg SP at $t=3$ h and liposomes containing 3.0 mg at $t=6$ h. At 15 min and 1, 2 and 3 h after each injection venous blood samples were taken and PBMC or plasma were isolated. Loading of PBMC with SP was assayed as described in the legends to Fig. 2. The results obtained with clone CAAp151-1 are shown as representative experiment. Normalization of the results was performed as described in Section 2.

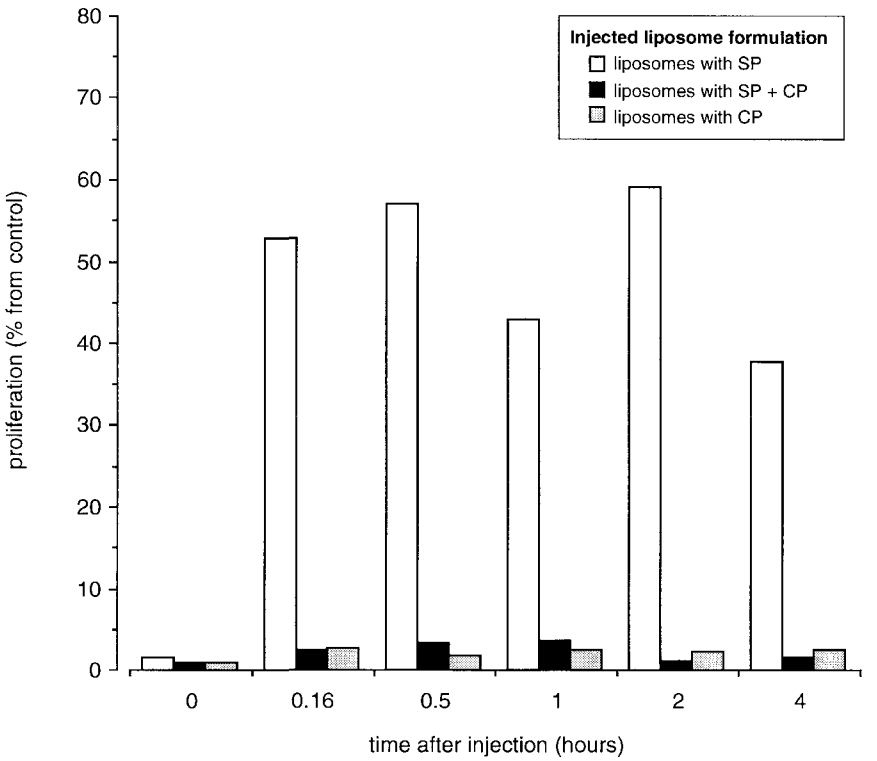

Fig. 4. Effect of incorporation of the competitor peptide (CP) in the liposomes. On 3 consecutive days three liposome formulations were tested, each given as 5 ml bolus injections. Liposomes containing only 0.3 mg SP/5 ml suspension were injected at day 1. Liposomes containing 0.3 mg SP and 30 mg CP were injected at day 2. Liposomes containing only 30 mg CP were injected at day 3. Loading of PBMC with SP was assayed by proliferation induction of clone DAAp151-1. Normalization of the results was performed as described in Section 2.

At 15 min, 1, 2 and 3 h after each injection, plasma and PBMC were isolated and tested for proliferation–induction of the clones (Fig. 3). Interestingly, already at a dose of 0.3 mg injected peptide significant proliferation of the clones was found. The maximal stimulatory capacity of the PBMC was found at 2 h after injection of the liposomes. Injection of liposomes with 1.0 or 3.0 mg SP resulted in higher and more sustained stimulatory capacity of the PBMC.

3.3. *Effect of CP on Mamu-DR3 binding of SP*

The results discussed thus far provide evidence that liposomes may serve as a vehicle for targeting of peptide to *MHC-DR* antigens on APC. The nonstimulatory CP p4-15(E > R) was found to abolish presentation of SP by competitive inhibition of binding to HLA-DR3 in vitro. *Mamu-DR3* loading with CP in vivo was assessed indirectly, namely by inhibition of SP presentation by PBMC to the T-cell clones. It is known that a 100-fold higher concentration of CP than of SP in the culture medium is needed for complete inhibition of T-cell proliferation. Hence, SP was encapsulated with 100-fold excess CP in the liposomes. Two monkeys were injected at 3 consecutive days with the peptide-containing liposomes and the stimulatory capacity of their PBMC was tested on clones CAAp151-1, DAAp151-1 and DAAp151-2 (Fig. 4). The figure shows that the sustained stimulatory activity of PBMC isolated after injection of liposomes containing only SP is completely abolished by encapsulation of excess CP in the liposomes. This suggests that CP binds effectively to the *Mamu-DR3* molecules and prevents binding of SP. As anticipated, no stimulatory activity of PBMC was found after injection of liposomes containing only CP.

3.4. *Conclusion*

Following the pioneering work of Adorini and coworkers on in vivo competition between self and foreign peptides for MHC binding [20], the use of synthetic peptides for specific therapy of autoimmune diseases has been assessed by many investigators. Promising data have been obtained especially in the two very well characterized murine autoimmune disease models, namely experimental autoimmune encephalomyelitis and collagen-induced arthritis [4,5,21–23]. The main conclusion of the present study is that liposome–peptide preparations can be used for efficient delivery of peptides to the relevant MHC class II molecules. The logic extension of our present study is to test if liposomes containing competitor peptides can be used to prevent or control disease in the rhesus monkey EAE model. Two main immune-response gene products, one *Mamu-DP* and one *Mamu-DR* molecule with their respective epitopes from the MBP molecule have been identified recently [12,24]. Such a study will reveal if peptide-mediated therapy of autoimmune disease is feasible in an outbred primate population and if a liposome formulation can overcome the continuous supply of high peptide doses which are necessary for sustained saturation of the target MHC class II molecules [13,25].

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