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ORIGINAL INVESTIGATION

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Induction of T cell response by bluetongue virus core-like particles expressing a T cell epitope of the M1 protein of influenza A virus

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Abstract A $CD4^+$ T cell epitope of the influenza virus matrix protein corresponding to the C terminus (QAYQKRMGVQMQRFK) was inserted into the VP7 gene of bluetongue virus (BTV). The chimeric protein was expressed by a dual recombinant Autographa californica polyhedrosis virus (AcNPV), which encodes the two inner capsid proteins VP3 and VP7 of BTV. When *Spodoptera frugiperda* cells (Sf9 cells) were infected with this recombinant BTV, core-like particles (CLPs) were formed as demonstrated by electron microscopy. To study the immunogenicity of a foreign epitope deprived of its natural flanking sequences in vitro, purified CLPs expressing the T cell epitope were used to stimulate two different MHC class II-restricted $CD4^+$ human T cell clones. One of these T cell clones, ALF 3.7 was specific for the inserted epitope, whereas the other T cell clone ALF 4.4 recognized shorter derivates of the given epitope. CLPs with the inserted epitope were presented as efficiently as purified influenza virus matrix protein to the clone ALF 3.7, whereas clone ALF 4.4 showed no proliferative response.

Key words T cell epitopes · Bluetongue virus · Core-like particles · Influenza virus M1 protein

Introduction

T cells recognize antigen only in the form of processed peptides bound to either MHC class I or class II molecules [1, 2]. Class II-bound peptides occur as nested sets derived from an epitope with variable truncations at both the N and C termini, and range from 9 to 30 amino acids in length. This antigen processing requires internalization of the antigen into an acidic compartment where the antigen is degraded and the resulting peptides are loaded onto MHC class II molecules. Subsequently, these complexes are displayed on the surface of the antigen-presenting cell (APC). Multiple factors influence a T cell response to an antigenic determinant, such as the affinity for the binding groove of the MHC molecule [3], competition between different peptides generated from the same antigen [4], and its availability after processing by APC [5]. The mechanisms of antigen processing remain unclear, but it is well established that the flanking sequences of a minimal antigenic determinant can influence its presentation to T cells [6–12]. Therefore, the immunogenicity of a given T cell epitope depends not only on its own sequence.

Over the last few years much effort has been focused on the development of synthetic peptide vaccines against various pathogens. One problem of peptide vaccines is their short lifetime. Another is the genetic restriction to the antigen which results in the failure to generate T cell help for antibody production. Thus, the identification of T cell determinants which are associated with protective immunity and can interact with a wide range of major histocompatibility complex (MHC) molecules is important in the design of synthetic vaccines. Therefore, it is first necessary to map T cell epitopes and second to look for a suitable delivery system of antigenic epitopes. Different types of vehicles have been used to present foreign B or T cell eptitopes, such as particles formed from hepatitis B virus (HBV) surface or core antigens, polioviruses, yeast Ty-particles or bacterial fusion proteins [13–17]. However, the processing and the presentation of such chimeric proteins is poorly documented.

In the present study, we have analyzed the immunogenicity in vitro of a $CD4^+$ T cell epitope of the matrix protein of influenza A virus which was genetically inserted into bluetongue virus (BTV, Orbivirus genus, Reoviridae) derived core-like particles (CLPs). Expression of both inner capsid proteins (VP3 and VP7) of BTV in insect cells via a dual recombinant baculovirus led to the formation of viral CLPs [18]. These particles can be easily purified by

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a one-step sucrose gradient centrifugation and it has already been shown that they work as an antigen delivery system for B cell epitopes [19]. Trimers of VP7 clothe an inner scaffold constructed of VP3 [20]. We have used an insertion site at amino acid (aa) position M145, which is exposed on the top of the VP7 molecule. The inserted $CD4^+$ T cell epitope is 15 aa long and corresponds to the C terminus of influenza virus matrix protein. The insertion had no influence on expression of the VP7 molecule, trimer formation or the generation of CLPs. The immunogenicity of CLPs carrying this viral epitope in vitro was investigated using two $CD4^+$ T cell clones which are matrix protein specific. One of these clones (ALF 3.7) was specific for the inserted epitope, whereas the other (ALF 4.4) recognized shorter derivates of the epitope. The expressed VP7 chimera showed a strong immunogenicity, comparable to that of purified influenza virus matrix protein, but interestingly no shorter derivates of this peptide were generated by APCs.

Materials and methods

Cells and viruses

The *Spodoptera frugiperda* clone Sf9 of IPLB-Sf21-AE cells [21] was maintained either as monolayer culture or as a suspension culture in TC 100 insect cell medium containing 10% FCS (Gibco BRL, Paisley, UK) as described previously [22].

Epstein Barr virus-transformed human B cells were maintained as a suspension culture in RPMI 1640 medium containing 10% FCS (Gibco) as described [23]. The HLA of these cells was typed as follows: A2 Aw26 Bw58 Bw49 DR6 DQ1.

Peripheral blood lymphocytes were stimulated with various dilutions of influenza virus matrix protein [strain A/FPV/Rostock/34 (H7/N1)]. The specificity of the resulting $CD4^+$ T cell clones was examined with overlapping petides synthesized by the SPOT method [24]. They were maintained as monolayer cultures in RPMI 1640 medium containing 5% human AB serum (Sigma-Aldrich, Deisenhofen, Germany) supplemented with 100 U/ml human recombinant IL-2 as previously described [25].

DNA manipulation and construction of a dual transfer vector carrying a T cell epitope

DNA modifications and subcloning of DNA were performed according to standard molecular procedures [26]. The dual baculovirus transfer vector pAcUW51-17.3-10.7 (M145), which expresses BTV protein VP3 under the control of the p10 promoter and BTV VP7 under the control of the polyhedrin promoter carries an insertion site for foreign epitopes in the context of VP7 with the restriction enzyme sites *Spe*I at the 5' end and *Sma*I at the 3′ end. The protein coding sequence of the chosen epitope QAYQKRMGVQMQRFK of influenza virus matrix protein was amplified by the polymerase chain reaction (PCR) using the oligonucleotide CTTCTTTCTAGAATGCAGGCCTACCAG as a 5′-forward primer and the oligonucleotide ATAATGAGACCCGGGCTTGAATCGTTG as 3′-reverse primer. The forward primer contained a *Xba*I-restriction site, and the reverse primer contained a *Sma*I-restriction site. Amplification was performed using a commercially available kit from Perkin Elmer Cetus Instruments (Weiterstadt, Germany). The amplified PCR product was digested with *Xba*I and *Sma*I and subsequently ligated into the *Spe*I and *Sma*I site of the baculo transfer vector pAcUW51-17.3-10.7 (M145) resulting in the plasmid pAcUW51/M-COOH. The orientation of the chimeric gene in the transfer vector was confirmed by sequence analysis [26].

Monolayers of *S. frugiperda* cells were co-transfected with pAcUW51/M-COOH and AcRP23.lacZ baculovirus DNA (Dianova, Hamburg, Germany) using the lipofectin reagent (BRL Life Technologies, Gaithersburg, Md.) [28, 29]. Recombinant baculoviruses generated by homologous recombination were selected by plaque formation on X-Gal plates. By plaque purification of progeny virus with a lacZ-negative phenotype the recombinant AcBTV17.3–10.7/M-COOH virus was isolated and high-titered virus stock was prepared.

Metabolic radiolabeling and immunoprecipitation of BTV VP3 and VP7

S. frugiperda cells were infected at a multiplicity of infection (m.o.i.) of 4 PFU/cell with either recombinant baculoviruses or were mock infected. At 2 days after inoculation the cells were washed once with methionine- and tryptose-free medium and preincubated with this medium for 1 h. Subsequently, the cells were labeled for 1 h with [^{35}S]methionine (Amersham/Buchler, Braunschweig, Germany) (10 μCi/ml) in TC 100 medium lacking the respective cold amino acid. After washing the cells twice in cold phosphate-buffered saline (PBS), they were solubilized in radioimmunoprecipitation buffer (RIPA; 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 10 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 500 U aprotinin, 20 mM TRIS hydrochloride pH 7.6). The lysates were centrifuged to remove insoluble debris, and the proteins were precipitated with anti-BTV hyperimmune rabbit serum and preswollen protein A-conjugated Sepharose 4B (Sigma). The precipitates were washed extensively with RIPA buffer. Protein dissociation buffer (10% mercaptoethanol, 10% sodium dodecyl sulfate, 25% glycerol, 10 mM TRIS hydrochloride pH 6.8, 0.02% bromophenol blue) was added to each sample, and heated to 100°C for 5 min. Proteins were analyzed by SDS polacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gels [30].

Purification of CLPs expressed in insect cells

For the purification of expressed particles, 3×10^8 Sf9 cells (spinner cultures) were infected at a m.o.i. of 4 PFU/cell with either the recombinant baculovirus AcBTV17.3–10.7/M-COOH or AcBTV17.3-10.7. At 48 h post infection the cells were harvested, washed with PBS, and lysed for 4 h at 4°C in 50 mM TRIS hydrochloride pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40. The cell lysates were centrifuged to remove insoluble debris, and the resulting supernatants were banded on a discontinuous sucrose gradient [30%:50% (wt/vol) in 0.2 M TRIS hydrochloride pH 8.0] after centrifugation at 85 000 *g* for 3 h. The resulting purified CLPs were analyzed by electron microscopy.

Electron microscopy

Purified particles were absorbed onto copper 400-mesh Formvar carbon-coated grids by floating the grids on droplets of the material for 2 min. After being washed twice in 0.2 M TRIS hydrochloride pH 8.0, the particles were stained for 20 s on droplets of 2% uranyl acetate. All grids were examined in a Zeiss EM 109 electron microscope.

Proliferation assay

T cells (2×10^4–3×10^4 cells) and mitomycin C-treated autologous transformed B cells (2×10^4 cells) in RPMI 1640 medium containing 10% FCS were plated in flat-bottom microtiter plates and incubated with various concentrations (1–100 μg/ml) of influenza virus matrix protein or CLPs. Matrix protein was purified from concentrated virus suspensions by lysis with 2% SDS followed by gel filtration [24]. After 48 h of incubation at 37°C 1 μCi [^{3}H]thymidine (Amersham/Buchler) was added to each well, and the cultures were pulsed for 18 h. Cells were harvested on fiberglass filters, and [^{3}H]thymidine incorporation was measured by liquid scintillation counting.

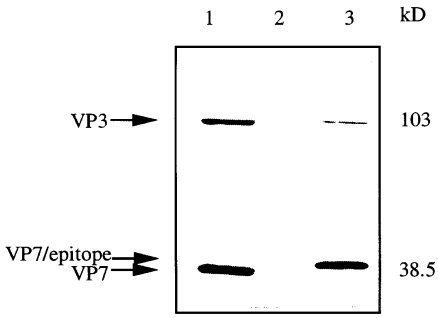

Fig. 1 Identification of BTV VP3, VP7 and VP7 with an inserted T cell epitope by metabolic labeling. Sf9 cells were infected with AcBTV17.3-10.7(M145) (*lane 1*), mock-infected cells (*lane 2*), and with AcBTV17.3-10.7/M-COOH (*lane 3*) for 48 h. The infected cells were labelled by a 2-h pulse with [^{35}S]methionine, and the proteins immunoprecipitated using an anti-BTV immune serum and analyzed by SDS-PAGE. Protein bands were visualized by fluorography (*BTV* bluetongue virus)

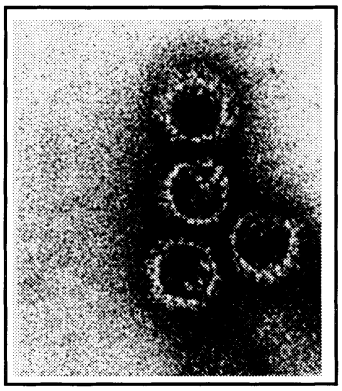

Fig. 2 Electron microscopy of purified CLPs obtained from Sf9 cells infected with AcBTV17.3-10.7/M-COOH (*CLP* core-like particle) ×30 000

Results

Expression of the BTV proteins VP3 and VP7 and the VP7-M1 chimera

Complementary DNA coding for the C-terminal peptide QAYQKRMGVQMQRFK of influenza virus matrix protein was cloned into the VP7 gene of BTV in the dual baculovirus transfer vector pACUW51-17.3-10.7 (M145), which encodes both BTV inner core proteins (VP3 and VP7) (for details see Materials and methods). Monolayers of *S. frugiperda* cells were infected with either the dual recombinant baculovirus AcBTV17.3-10.7 or AcBTV17.3-10.7/M-COOH at a m.o.i. of 4 PFU. Two days after inoculation the Sf9 cells were labeled with [^{35}S]methionine for 1 h. Antigens from the cell lysates were immunoprecipitated with an anti-BTV hyperimmune rabbit serum. Labeled proteins were separated by SDS-PAGE and visualized by fluorography. Both the modified and unmodified VP7 proteins reacted with the anti-BTV serum (Fig. 1). The VP7 chimera had an increased molecular weight reflecting the insertion of the 1.5-kDa epitope. No degradation products were detectable and the expression levels of the precipitated proteins were identical.

Preparation of BTV CLPs carrying a T cell epitope

To assess the ability of the dual recombinant baculoviruses to produce CLPs, suspension cultures of Sf9 cells were infected with either AcBTV17.3-10.3 or AcBTV17.3-10.7/M-COOH at a m.o.i. of 4 PFU. At 48 h post infection cells were lysed with the nonionic detergent Nonidet P-40, and the released particles were purified by centrifugation on discontinuous sucrose gradients. When the material obtained from the gradient was examined by electron microscopy, single-shelled CLPs were observed (Fig. 2). Polyacrylamide gel electrophoresis revealed that they were composed of VP3 and VP7. Comparison between wild-type CLPs and CLPs carrying the T cell epitope showed no differences (data not shown). This indicates that the insertion of the T cell epitope QAYQKRMGVQMQRFK within the context of BTV VP7 did not alter the folding or transport of this molecule, because otherwise VP7 trimer formation and CLP assembly would not have occurred [20].

Induction of proliferative T cell response by the M1 epitope expressed in BVT CLPs

The immunogenicity of the M1 sequence present in CLPs was examined in a T cell proliferation assay. The experiments were conducted using two M1-specific MHC class II-restricted T cell clones from one donor. These T cell clones had been generated by stimulation in vitro with purified matrix protein, and their recognized epitopes have been mapped with a series of overlapping synthetic peptides [24]. The epitope presented in the VP7 chimera is immunodominant for the T cell clone ALF 3.7, but not for the T cell clone ALF 4.4, which recognizes only shorter derivates of this peptide (Fig. 3). The T cell clones and autologous mitomycin C-treated transformed B cells were plated in 96-well flat-bottom plates and incubated with purified matrix protein, wild-type CLPs, and CLPs with the inserted T cell epitope. The proliferative T cell responses were measured by [^{3}H]thymidine incorporation 48 h after stimulation with the exogenous antigens. The experiments were carried out in triplicates. The proliferative responses of both T cell clones are shown in Fig. 4. Both clones reacted with their original antigen, the matrix protein, whereas no significant response was observed with wild-type CLPs. Interestingly, the inserted epitope was presented as efficiently as the purified matrix protein to the T cell clone ALF 3.7. However, clone ALF 4.4. showed no proliferative response to the chimeric CLPs. These results showed that the inserted epitope QAYQKRMGVQMQRFK is presented very efficiently to the specific T cell clone ALF 3.7. Thus

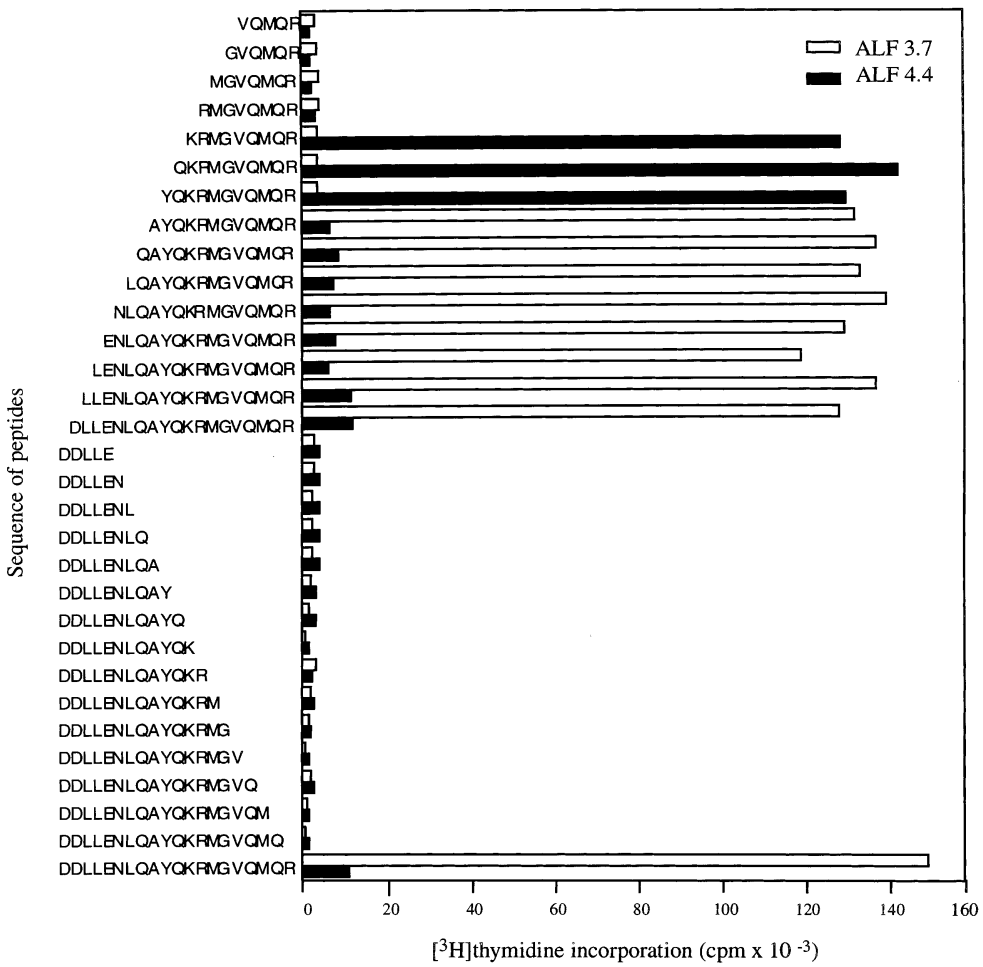

Fig. 3 Fine mapping of one DQ-restricted (ALF 3.7) and one DP-restricted (ALF 4.4) T cell clone by proliferation assay; 2×10^4 T cells and 3×10^4 mitomycin C-treated autologous transformed B cells in RPMI 1640 medium containing 10% FCS were plated in flat-bottom microtiter plates and incubated with CLPs (30 µg/ml). After 48 h of incubation incorporation of [^{3}H]thymidine was determined as described in Materials and methods

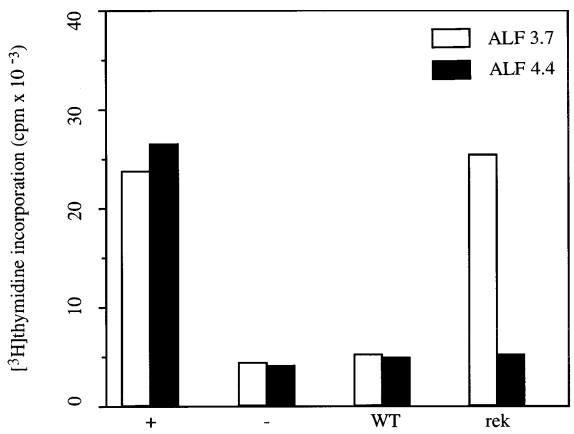

Fig. 4 Presentation of BTV derived CLPs by a EBV-transformed human B cell line. Mitomycin C-treated EBV cells were incubated with 10 µg purified matrix protein (+), without antigen (–), with 100 µg wild-type CLPs (*WT*) and with 100 µg CLPs expressing a chimeric VP7 molecule carrying the described T cell epitope (*rek*). Matrix protein-specific T cells were added for 48 h and pulsed with [^{3}H]thymidine for additional 18 h

processing of this epitope out of the context of the BTV VP7 was possible. However, as indicated by the unresponsiveness of ALF 4.4, further processing of the epitope did not occur.

Discussion

In the present study we have investigated the delivery of a peptide from exogenous sources to MHC class II molecules. The peptide derives from the influenza virus M1 protein, corresponds to the C-terminus of the molecule (QAYQKRMGVQMQRFK) and activates two CD4$^+$ T cell clones (ALF 3.7 and ALF 4.4). This epitope was genetically inserted at position 145 of BTV VP7 protein. Using a described genetic procedure to express foreign peptides in the context of VP7 [19], we have achieved the formation of CLPs with a chimeric VP7 molecule. In a functional assay of antigen presentation, this epitope is delivered effectively to the T cell clone ALF 3.7 either in the context of M1 protein or chimeric VP7. In contrast, clone ALF 4.4, which was assigned to recognize shorter derivates of the epitope (see Fig. 3), showed no proliferative response to CLPs but to M1 protein. These results indicate that the inserted epitope was efficiently presented to a specific T cell clone (ALF 3.7) but further processing, to achieve shorter antigenic peptides, did not occur. How can the present result be interpreted?

In contrast to MHC class I-bound peptides, which are predominantly nonamers [31–33], class II-bound peptides occur as nested sets derived from a single epitope with var-

ious truncations at both the N and C termini [34–36]. There must be an evolutionary advantage for the class II antigen-processing machinery to generate such nested sets of peptides. Viruses, for example, have been shown to escape immune elimination by the mutation of key T cell receptor contact residues [37]. The generation of multiple determinants from combining large nested sets of peptides with a single MHC class II molecule [38, 39] could, thus, significantly reduce viral escape. This reflects a dependence of the processing and presentation machinerie to flanking sequences of an immunodominant epitope to generate and select optimal fitting peptides from the large amount of peptides that are made by proteolysis of a protein. For the inserted M1 epitope an inhibitory effect of the flanking regions of VP7 can be supposed. The unresponsiveness of clone ALF 4.4 demonstrates that the processing of the M1 epitope inserted within the context of VP7 undergoes a different route of processing than the M1 protein. Further, it demonstrates that peptides generated from different forms of an external antigen (purified M1 protein, CLPs carrying a chimeric VP7 molecule) do not seem to have identical structures when associated with MHC class II molecules.

Different carriers like authentic M1 protein or CLPs also play a major role in the efficacy with which antigenic peptides are internalized, processed and subsequently routed by APCs. The normal way of antigen uptake by APCs is phagocytosis followed by processing of the antigen into short peptide fragments within the processing machinery of the cell. An alternative is receptor-mediated endocytosis. As reported by Grimes et al. [20], a conserved aa sequence Arg-Gly-Asp (168–170) is located within the VP7 molecule in an exposed position on the core. Such tripeptides are involved in many integrin-dependent cell adhesion processess. Thus, it can be hypothesized that this sequence is recognized by integrins on the cell surface of the APC which would act as a receptor for BTV CLPs. This complex is subsequently internalized via receptor-mediated endocytosis, the antigen is processed and the generated peptides are displayed on the surface of the APC. Provided the two described ways represent the mode of entry of CLPs into the processing machinery of APCs, it would be reasonable to expect degradation of the inserted epitope as it takes place within the M1 protein where series of peptides are generated with scattered ends. The unresponsiveness of clone ALF 4.4 challenged with chimeric CLPs demonstrated that an alternative way of antigen uptake or antigen processing exists.

The insertion site of the epitope within the VP7 molecule is located in an exposed loop and sticks out of the protein. Thus, it can be hypothesized that CLPs with an inserted epitope can directly bind to empty MHC class II molecules on the surface of APCs. This implies that the MHC molecule acts as receptor for CLPs if the epitope has an high affinity to the MHC binding groove. After internalization the MHC-CLP complex follows the pathway of MHC class II recycling but the epitope may be protected against further degradation by cellular proteases and subsequently no shorter derivates of the inserted epitope, which are determinants for the clone ALF 4.4, are generated. This possibility is supported by the finding that a 34-aa hen eggwhite lysozyme peptide, once bound to the I-A^b molecule, becomes inaccessible to proteolytic degradation [40].

The introduced CLPs are interesting candidates for antigen delivery. The particles are composed of 780 copies of VP7 within antigenic determinants (B cell epitopes as well as T cell epitopes) which can be expressed, so that large amounts of immunogenic epitopes are presented to the immune system. They can be easily purified in large amounts, induce T cell proliferation and are highly immunogenic, as shown by Belyaev and Roy [19]. For future work it would be interesting to look for the immunogenicity of longer epitopes than the minmal epitope or to express several copies of a given antigenic determinant within CLPs. Using single expression vectors it will be possible to express different combination of B and T cell epitopes within one CLP.

These data indicate that BTV CLPs represent effective carriers for delivery of peptides to MHC class II molecules, applicable for vaccination or tolerization protocols. They have the potential to be long-lived and to be devoid of side effects.

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