A Novel CD4⁺ T-Helper Lymphocyte Epitope in the VP3 Protein of Hepatitis A Virus

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Prediction analysis of T₄-cell epitopes in the VP3 capsid protein of the hepatitis A virus (HAV) revealed the occurrence of a putative T₄ epitope in the 102–121 region complying with all the algorithms tested. To confirm these predictions, spleen T lymphocytes obtained from BALB/c mice previously immunised with HAV, were stimulated in vitro with different concentrations of synthetic peptides 102–121 and 110–121 of VP3. The ability of these peptides to stimulate CD4⁺ T-helper lymphocytes proliferation was evaluated by an immunological flow cytometry detection of bromodeoxyuridine incorporation. Using this method, it is concluded that the amino terminal part of the VP3 102–121 sequence contains a T cell epitope.

INTRODUCTION

The induction of specific CD4⁺ T-helper (T₄) lymphocytes proliferation in response to antigens is of critical importance for an efficient antibody response.

Antigens are processed by the antigen-presenting cells (APCs) into peptide fragments which are presented on the cell surface of APCs in association with MHC class II molecules. The interaction of the peptide bound to the MHC class II molecule with the T cell receptor (TCR) of T₄ lymphocytes stimulates its proliferation. These T₄ lymphocytes secrete cytokines, which mediate a variety of immune responses, including the activation of antigen specific B lymphocytes. The localisation of T₄ epitopes corresponding to the peptide fragments which bind to the MHC class II molecules in viruses is of great important in the design of synthetic vaccines.

In picornaviruses, characterisation of T₄ epitopes has been reported for poliovirus [Leclerc et al., 1991; Kutubuddin et al., 1992; Mahon et al., 1992; Graham et al., 1993], human rhinovirus [Hastings et al., 1993], food-and-mouth disease virus (FMDV) [Rodriguez et al., 1994], Mengo virus [Muir et al., 1994] and Theiler’s virus [Usherwood et al., 1995].

In the case of HAV, putative T₄ epitopes in the VP2 region [Powdrill and Johnston, 1991] and in the carboxy terminal region of VP1 [Harmon et al., 1993] have been described. Later on, two peptides derived from the VP1 region, 17–33 and 276–298, were demonstrated to stimulate T lymphocytes of several mouse haplotypes [Ivanov et al., 1994]. In the same study, peptides 45–57 and 137–150 derived from the VP3 protein were predicted as T-epitopes by a combination of different computational methods, but only the peptide 137–150 was able to stimulate one of the mouse haplotypes tested.

In a previous study [Bosch et al., 1998], a continuous B-cell epitope was described in the VP3 protein of HAV at position 110–121. In order to improve the immunological response against a synthetic peptide containing this B-epitope, a longer peptide was subsequently synthesised corresponding to the sequence VP3 102–121. The anti-HAV titre achieved with the VP3(102–121) peptide was 100 times higher than the response obtained with the VP3(110–121), although the recognition of both peptides by a human convalescent serum was similar [Pintó et al., 1998]. Taken together, these results suggested the presence of a putative T-epitope in the VP3 102–121 sequence. In the present study, the ability of the peptide VP3(102–121) to induce proliferative responses of T₄ lymphocytes from BALB/c mice primed with HAV was evaluated, in order to confirm the occurrence of a T₄-epitope in this sequence.

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MATERIALS AND METHODS

Viruses and Cells

The cytopathogenic HM-175 strain of HAV (courtesy of T. Cromeans, Centers for Disease Control, Atlanta, GA) [Cromeans et al., 1987] was propagated in FRhK-4 cells. Concentrated viral stocks were obtained as described previously [Bishop et al., 1994]. Briefly, at 5–6 days post-infection cells from a T-175 cm² flask were recovered by trypsin treatment, collected by centrifugation, resuspended in 500 µl of NT buffer (0.1 M NaCl, 10 mM Tris-HCl, 1% NP40, pH 7.4) and incubated for 30 min. These lysed cell suspensions were centrifuged at 1,700g for 5 min, and the supernatants again centrifuged at 13,000g for 5 min. Viruses recovered in the supernatants were submitted to three sonication cycles of 30 sec at 60 W in the presence of 0.4% SDS. Five hundred microlitres of these NP-40 stocks were layered onto a 5–45% sucrose gradient in TNMg buffer (20 mM Tris-HCl, 10 mM NaCl and 50 mM MgCl₂, pH 6.7) and spun at 205,000g for 165 min.

Fractions containing infectious virus (150S) and empty particles (70S) were pooled and dialysed against phosphate buffered saline (PBS) to remove sucrose. After 3 days of dialysis, the pools were concentrated by centrifugation through Centriplus filters Y-50 (Millipore Corp., Bedford, MA) following the manufacturer’s instructions.

Prediction of T Cell Epitopes in the VP3 Protein

The computer program Epiplot 1.0 [Menéndez-Arias and Rodriguez, 1990] was used to examine the VP3 aminoacid sequence of HM-175 strain [Cohen et al., 1987] for prediction of T₁₁ epitopes. It is based on four different algorithms; the Fauchere–Pliska hydrophilicity scale [Faubchere and Pliska, 1983] with a block length of 11 and an amphipathicity score threshold of 8; the occurrence of Rothbard–Taylor motifs recognised by MHC class II molecules [Rothbard and Taylor, 1988]; the search for sequences with analogy to ovalbumin peptides which are known to bind to the murine MHC class II (Ia) molecules [Sette et al., 1989]; and the hydrophobic strip-of-helix algorithm with three turns per helix [Stille et al., 1987].

The amphipathic plot of VP3 was predicted using the WinPep program [Hennig, 1999] available at http://www.ipw.agrl.ethz.ch/~hennig/winpep.html. Helical wheel representations of the VP3 102–114 and VP3 110–121 sequences were also drawn with the WinPep program and the hydrophobic character of residues was calculated according to a hydrophobicity scale described previously [Black and Mould, 1991].

The consensus aminoacid secondary structure prediction was undertaken using the Network Protein Server Analysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html) [Combet et al., 2000].

Synthetic Peptides

Peptides corresponding to the VP3(102–121) (ASICQMFCFWRGDLVDFQV) and VP3(110–121) (FWRGDLVDFQV) sequences (kindly provided by Dr. Isabel Haro from the Department of Peptides, CID, CSIC, Barcelona, Spain) were synthesised by the continuous-flow F-moc-polyamide solid-phase method [Stewart, 1983]. Peptides were purified to a purity greater than 95% by semi-preparative HPLC.

Immunization of Mice

Three 6-week-old female BALB/c (H-2b) mice (Harlan Interfauna Ibérica, Barcelona, Spain) were injected subcutaneously with 3.7 ng of virus particles, diluted 1:2 in Freund’s complete adjuvant, in a final volume of 0.2 ml. A booster dose in Freund’s incomplete adjuvant was administered 2 weeks later. Three mice inoculated with 0.2 ml of PBS were used as negative controls.

Animals were handled and killed according to specific guidelines of the Animal Ethics and Experimentation Committee of the University of Barcelona. All experiments were repeated three times.

Lymphocyte Proliferation Assays

One week after the booster dose, spleens from three immunised mice were aseptically removed, pooled in RPMI 1640 medium containing 10% foetal calf serum (FCS) and mechanically homogenised. A single cell suspension was prepared by filtering through an 80-mesh screen (Sigma, St. Louis, MO).

T lymphocytes were purified using nylon wool fibre columns (Polysciences Europe, Eppelheim, Germany) following the manufacturer’s instructions. Briefly, after the column incubation with RPMI medium, the cell suspension was layered onto the column and was incubated for 1 hr at 37°C. Nonadherent T cells were then collected by using two washes. Accessory cells, to be used as antigen-presenting cells, were obtained from spleens of non-immunised mice and treated with mitomycin C [Coligan et al., 2001].

Proliferation assays were carried out in 96-well tissue culture plates seeded with 5 × 10⁵ purified T lymphocytes and 5 × 10⁴ antigen-presenting cells per well, in a final volume of 200 µl. Different concentrations of peptide antigens (0.05, 0.5 and 5 µM) were added and cultures were incubated for 6 and 10 days. Each condition was assayed in triplicate cultures. In all experiments the non-specific mitogen concanavalin A (Con A) at a concentration of 10 µg/ml was used as a positive control, and cultures receiving only culture medium were used as negative controls.

Cellular proliferation was measured by the incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA during the last 16 hr of culture until the harvesting of the cells. The BrdU was used at the experimentally determined optimal concentration of 60 µM.

Following the incorporation of BrdU, cells from three equivalent wells were mixed, washed twice in PBS...
containing 2% of BSA and 0.1% of Tween 20 (washing PBS) and labelled using a phycoerythrin (PE)-coupled anti-CD4 antibody (Becton Dickinson, San Jose, CA) for 30 min, at room temperature (RT). Cells were subsequently rinsed twice in washing PBS, resuspended in 0.5 ml of ice-cold 0.15 M NaCl, fixed with 1.2 ml of ice-cold ethanol and held for 30 min on ice. Cells were then washed and permeabilized in PBS-1% paraformaldehyde-0.01% Tween 20 for 1 hr at RT. Cells were washed again and incubated with 50 kunits U/ml of DNase for 30 min at 37°C. After further washing with PBS containing 10% BSA and 0.5% Tween 20, samples were incubated with 10 μl of an anti-BrdU-FITC antibody (Becton Dickinson) for 2.5 hr at 37°C, washed again with PBS and analysed by flow cytometry (Beckman Coulter XL, Miami, FL).

**Flow Cytometry Analysis**

In order to select the intact cell population, the forward-angle and the side-angle light scatter were used to select cell size and cell shape respectively.

Three different representations were obtained (Fig. 2). An histogram representing the FITC fluorescence which corresponds to the BrdU labelling, an histogram representing the PE fluorescence which corresponds to the CD4⁺ labelling and a dot-plot of the FITC fluorescence plotted versus the PE fluorescence. A cursor D was defined to include proliferative cells incorporating BrdU in its DNA, a cursor E was defined to select CD4⁺ lymphocytes, and both cursors were used to define the quadrant R2 which includes the subpopulation of proliferative CD4⁺ lymphocytes among the complete cell population. All these cursors were established in comparison to negative control cell populations. Cursor D was established as described previously [Abad et al., 1998]. Briefly, an arbitrary cursor B was drawn at the right end of the fluorescent curves of the negative controls, including 2% of the cell population. The mean fluorescence of cells within cursor B was calculated for each of the negative controls, and a new cursor D was established starting at a cut-off level corresponding to the mean plus 2 standard deviations of these mean fluorescence figures of the negative controls. Cursor E was arbitrarily drawn so to start at the inflexion point between the two curves representing CD4⁺ lymphocytes and other T lymphocytes.

The stimulation index (SI) was calculated dividing the percentage of cells in the R2 quadrant of the peptide or Con A stimulated cultures by the percentage of cells in the R2 quadrant of the medium stimulated cultures. In previous studies the stimulation response was considered significant when SI was higher than 2, 2.5 or 3 [Ivanov et al., 1994; Muir et al., 1994; Rodrı́guez et al., 1994].

**RESULTS AND DISCUSSION**

**Prediction of T Cell Epitopes in the VP3 Protein**

A high percentage of T⁺-cell epitopes are located within amphipathic helices [Bérzofsky, 1988], therefore different informatic programs were used for prediction of these structures in the VP3 capsid protein.

The Epiplot program was used in order to locate the putative T⁺-cell epitopes in the VP3 region. The results obtained with the different tested algorithms are summarised in Table I. Using these algorithms several potential T epitopes were located in the VP3 protein, particularly, at positions 96–114, 153–171 and 210–218.

In the predicted amphipathic profile of the VP3 protein, the regions 62–73, 96–109, 166–190 and 202–211 showed the higher amphiphathic index (Fig. 1A), and α-helices structures were located in regions 18–28 and 98–107 of the VP3 protein (Fig. 1B).

<table>
<thead>
<tr>
<th>VP3 segments</th>
<th>Fauchere–Pliska algorithm</th>
<th>Rothbard–Taylor motifs</th>
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TH-cell epitopes have been observed in several picornaviruses and it has been suggested a requirement of spatial proximity for an efficient antibody production [Kutubuddin et al., 1992; Graham et al., 1993; Muir et al., 1994].

This potential TH-cell epitope in the VP3 94–114 region of HAV aligns in part with an actual TH-cell epitope of FMDV [Usherwood and Nash, 1995] and completely with a putative TH-cell epitope of Mengo virus [Muir et al., 1994], as can be seen in Figure 1C. In order to confirm the actual occurrence of this TH-cell epitope, two synthetic peptides including the T-B cell epitopes were synthesised. The synthetic peptide VP3(110–121) includes the B-cell epitope and only a minor part of the putative TH-cell epitope in spite of its complete alignment with the actual FMDV TH-cell
epitope (Fig. 1C). However, structurally this sequence did not align with an α-helix but with a β-sheet (Fig. 1D). In contrast, the synthetic peptide VP3(102–121) includes a major part of the predicted TH-cell epitope and the B-cell epitope, and partially aligns with an α-helix (Fig. 1D).

The helical wheel representation of the peptide sequences corresponding to the theoretic TH-cell epitope

Fig. 2. Dot-plots and histograms of T lymphocytes cultured for 10 days after in vitro stimulation with different products. T lymphocytes from mice primed with HAV particles were stimulated with culture medium (A), concanavalin A (B) or synthetic peptide VP3(102–121) (C). T lymphocytes from mice inoculated with PBS were stimulated with the synthetic peptide VP3(102–121) (D). The numbers in the dot-plots show the percentage of cells within each quadrant.
(102–114) and the B-cell epitope (110–121), demonstrated an amphipathic structure, with the hydrophobic residues located on one side and the hydrophilic residues on the opposite side of the wheel, only for the sequence VP3 102–114 (Fig. 1E).

### Proliferative Response of HAV-Primed Lymphocytes to Synthetic Peptides

The lymphoproliferative activity of the synthetic peptides was assessed in spleen cells obtained from BALB/c mice injected with 3.7 ng of purified HAV particles or PBS (negative control). Lymphocytes were stimulated in vitro with 0.05, 0.5 and 5 μM of the synthetic peptides VP3(102–121) and VP3(110–121) for 6 and 10 days. Lymphocyte proliferation was estimated through the incorporation of BrdU in DNA, which was measured by flow cytometry (Fig. 2). A cursor D was defined to include the proliferative cells, as can be observed in the positive control (Fig. 2B) corresponding to Con A stimulated lymphocytes from HAV-primed mice, and negative controls (Fig. 2A,D) which correspond to culture medium stimulated lymphocytes from HAV-primed mice and VP3(102–121) stimulated lymphocytes from mice inoculated with PBS. Specific CD4+ lymphocytes were detected by an immune flow cytometry method based on the specific recognition of the CD4 receptor. Again a cursor E was defined to separate CD4+-expressing lymphocytes. Finally, the subpopulation of double labelled cells, quadrant R2 in the dot plots of Figure 2, was selected on the basis of the above mentioned cursors. Data from a representative experiment are shown in Figure 2, in which the percentage of proliferative CD4+ lymphocytes at 10 days after stimulation in the R2 quadrant is 0.51% (Fig. 2A), 6.82% (Fig. 2B) and 4.14% (Fig. 2C) in culture medium, Con A and VP3(102–121) stimulated lymphocyte cultures from HAV-primed mice, respectively, and 0.09% (Fig. 2D) in VP3(102–121) stimulated lymphocytes from mice inoculated with PBS.

The mean percentage of proliferative cells (Fig. 3A), proliferative CD4+ T lymphocytes (Fig. 3B) and CD4+ T lymphocytes (Fig. 3C) were estimated from three separate experiments. These percentages are the result of subtracting the percentage of cells from mice inoculated with PBS to the percentage of cells from mice primed with virus particles.

Interestingly, the percentage of total proliferative lymphocytes (Fig. 3A) and the percentage of specific proliferative CD4+ T lymphocytes (Fig. 3B) was equivalent in all the treatments, suggesting that CD4+ T lymphocytes were responsible for most of the proliferative responses.

![Fig. 3. Mean percentage of proliferative T lymphocytes (A), proliferative CD4+ T lymphocytes (B) and CD4+ T lymphocytes (C) after in vitro stimulation, of T lymphocytes obtained from HAV-primed mice, with culture medium (control), concanavalin A (Con A) or VP3-derived peptides. The cell percentage in D (A) and E (C) cursors and R2 quadrant (B) for each treatment was calculated by subtracting the percentage of cells from mice primed with PBS to percentage of cells from mice primed with virus particles.](image-url)
After 6 days of stimulation with the peptide VP3(102–121), the percentage of proliferative CD4+ lymphocytes in the quadrant R2, was directly proportional to the concentration of peptide and consequently the maximum proliferation was achieved at the 5 μM concentration (Fig. 3B). In contrast, no proliferative CD4+ lymphocyte response was observed with the synthetic peptide VP3 110–121 (Fig. 3B and Table II). Con A was used as a positive control, even though after 6 days of culture the percentage of proliferative CD4+ lymphocytes was not significantly different from that of the negative controls (Fig. 3B and Table II).

The percentage of proliferative CD4+ lymphocytes after 10 days of stimulation with both peptides was higher than that resulting after 6 days (Fig. 3B and Table II). At this same time (10 days post stimulation), Con A was able to induce CD4+ lymphocyte proliferation.

The SI, defined as the ratio between the percentage of proliferative CD4+ lymphocytes stimulated with the antigen and the percentage of proliferative CD4+ lymphocytes stimulated with culture medium, obtained with the different treatments are shown in Table II. Assuming a cut-off value of 3 [Muir et al., 1994], it is concluded that at 6 days of stimulation only the peptide VP3(102–121) showed proliferation activity, while at 10 days also the peptide VP3(110–121) and Con A induced proliferation, although at a lower level.

The occurrence of a T\textsubscript{H}-cell epitope is therefore confirmed in the sequence 102–114 of the VP3 protein of HAV, which overlaps contiguously with a well-characterised B-cell epitope in the sequence 110–121 [Bosch et al., 1998]. Although the putative T\textsubscript{H}-cell epitope is located at the sequence 96–114, the ability of a peptide starting at position 102 to induce the proliferation of primed CD4+ lymphocytes has been demonstrated in the present study. However, any potential synthetic vaccine should include an elongated carboxy end of the above mentioned B-epitope, since truncated carboxy peptides such as those corresponding to the sequences 110–117 and 110–119 are not recognised by convalescent sera [Bosch et al., 1998] and carboxy elongated forms of this sequence are better recognised by convalescent sera [Khudyakov et al., 1999].


