Identification of an HLA-A*0201-restricted cytotoxic T-lymphocyte epitope in rotavirus VP6 protein

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The function of cytotoxic T lymphocytes (CTLs) in rotavirus (RV) infection in humans is poorly understood. To date, no RV-specific human leukocyte antigen (HLA) class I-restricted T-cell epitopes have been described. In this study, four peptides derived from human RV Wa strain VP6 protein were predicted by computer algorithms and verified by an HLA*0201-binding assay. Two peptides with high affinity for HLA-A*0201 molecules were further assessed. The CTLs induced in vitro by P340–348 (TLLANVTAV)-loaded autologous dendritic cells from peripheral blood lymphocytes of HLA-A*0201-matched healthy donors released gamma interferon specifically upon stimulation with P340–348-loaded T2 cells. The CTLs lysed both P340–348-loaded T2 cells and human RV Wa strain-infected HLA-A*0201 + Caco-2 cells in an antigen-specific and HLA-A*0201-restricted manner. At the same time, P340–348 was shown to be immunogenic in vivo in HLA-A*0201/Kb transgenic mice. It is proposed that P340–348 is an HLA-A*0201-restricted CTL epitope.

To date, no effective and safe rotavirus (RV) prophylaxis vaccines are licensed for group A RVs. Better knowledge of the immune-protection mechanisms against RV in humans will be very useful for ongoing vaccine-development efforts. Experiments in animal models have demonstrated consistently that CD8+ T cells can mediate clearance of RV infection, as well as providing almost-complete short-term and partial long-term protection from reinfection (Offit & Dudzik, 1990; Franco et al., 1997; McNeal et al., 1997; Ward, 2003). However, the frequencies of virus-specific CD8+ T cells secreting gamma interferon (IFN-γ) after RV infection in children are undetectable or very low using an intracellular cytokine assay and ELISPOT assay (Jaimes et al., 2002; Rojas et al., 2003). Even in symptomatic RV-infected adults, the frequencies of these cells are relatively low compared with the frequencies of T cells specific for other pathogens (Jaimes et al., 2002; Rojas et al., 2003). There is the hypothesis that only a small percentage of circulating RV-specific T cells secrete cytokines, or that they secrete unique cytokines in an intestinal priming environment. An important step towards answering these and other questions concerning the role of cytotoxic T lymphocytes (CTLs) in RV infection is the identification of RV-specific human leukocyte antigen (HLA) class I-restricted CD8+ T-cell epitopes. Data from animal models have shown that VP6 is one of the main target antigens of RV-specific CD8+ T cells (Franco et al., 1994; Offit et al., 1994). The amino acid sequence of the VP6 protein is highly conserved (87–99%) among group A RVs in mammals (Tang et al., 1997), which suggests that a CTL response against VP6 protein would have the potential ability to protect the host against a broad variety of virus strains. Moreover, HLA-A2 is the most common HLA phenotype found in various ethnic populations. Among them, HLA-A*0201 is the predominant subtype (Player et al., 1996; Shieh et al., 1996). To develop new reagents for future studies on the role of CTLs in protection against RV and peptide-based vaccines, we decided to identify HLA-A*0201-restricted CTL epitopes derived from the VP6 protein of human RV Wa strain.

A computer-based program, accessed through SYFPEITHI (http://www.syfpeithi.de/scripts/MHCServer.dll/home.htm), was utilized to predict HLA-A*0201-restricted CTL epitopes within the VP6 protein of human RV strain Wa (GenBank accession no. P03530). Four candidate nonameric peptides with the highest scores were selected. These peptides were synthesized at Sangon (Shanghai, China) and purified to > 95 % by reverse-phase high-performance liquid chromatography, as verified by mass spectrometry. To determine the binding ability of the candidate peptides to HLA-A*0201 molecules, 20 μM peptide and 5 μg human β2-microglobulin ml−1 (Serotec) were incubated with the transporter associated with antigen presentation-deficient T2 cells in serum-free RPMI 1640 medium for 18 h at 37 °C. The T2 cells were then stained with anti-HLA-A2 mAb BB7.2 and incubated with fluorescein isothiocyanate...
HLA-A*0201–peptide complexes was assessed as described (FI 2 peptides, P340–348 (TLLANVTAV) and P333–341 fluorescence without peptide)/(median FITC fluorescence without peptide). Among the four candidate FITC fluorescence without peptide)/(median FITC fluorescence without peptide). Among the four candidate control peptides, P340–348 (TLLANVTAV) and P333–341 (VLADANETL) were shown to be low-affinity peptides (FI < 1:5), whilst the positive-control peptide HBCag18–27 and the negative-control peptide HBCag131–140 had FI ratios of 2:36 and 0:18, respectively. The ability of these peptides to form stable HLA-A*0201–peptide complexes was assessed as described by Passoni et al. (2002). Briefly, after incubation with 20 μM peptide in serum-free RPMI 1640 medium supplemented with 100 ng human β2-microglobulin ml−1 for 18 h, T2 cells were washed to remove free peptides. Then, 10 μg brefeldin A ml−1 (Sigma-Aldrich) was added to block cell-surface expression of newly synthesized HLA-A*0201 molecules and incubated for 1 h. The T2 cells were washed and incubated at 37 °C for 0, 2, 4, 6 or 8 h. For each time point, peptide-induced HLA-A*0201 expression was calculated as Fl. Dissociation complex 50 (DC 50) was defined as the time required for the loss of 50% of the HLA-A*0201–peptide complexes stabilized at t ¼ 0. P340–348 and P333–341, with strong binding ability to HLA-A*0201 molecules, were also shown to be efficient stabilizers (DC 50 > 8 h), which suggests good potential immunogenicity, and were selected for further testing.

To investigate the capacity of P340–348 (TLLANVTAV) and P333–341 (VLADANETL) to mobilize a human CTL repertoire, peptide-specific CTLs were generated by in vitro sensitization of healthy HLA-A*0201+ donor peripheral blood lymphocytes (PBLs) with autologous dendritic cells (DCs) pre-pulsed with P340–348 and P333–341. PBLs were obtained from the non-adherent cells of peripheral blood mononuclear cells (PBMCs) that were incubated for 2 h at 37 °C in six-well plates and frozen. The PBMCs were isolated from the whole blood of healthy HLA-A*0201+ volunteer donors by using Ficoll/Hypaque (Axis-Shield PoC) density-gradient centrifugation. Human peripheral blood monocyte-derived DCs were generated as described by Sallusto & Lanzavecchia (1994). Briefly, the adherent monocyte-enriched PBMCs were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 20 ng recombinant human interleukin (IL)-4 ml−1 (R&D Systems) and 20 ng recombinant human granulocyte–macrophage colony-stimulating factor ml−1 (R&D Systems). On day 5, 10 ng recombinant human tumour necrosis factor alpha ml−1 (R&D Systems) was added to the medium to induce phenotypic and functional maturation. On day 7, mature DCs were harvested, pulsed with 10 μM peptide for 3 h at 37 °C and irradiated at 3000 rad. Then, peptide-loaded irradiated DCs were co-cultured with autologous PBLs in the presence of 10 ng recombinant human IL-7 ml−1 (Peprotech Inc.). The responder: stimulator cell ratio was 10:1 (PBLs: DCs) for priming and 20:1 for restimulation. After the PBLs were primed for 7 days, a standard 4 h 51Cr assay (Amersham Biosciences) was carried out to detect PBL activity against RV-infected Caco-2 cells and non-detectable CTL activity was observed (Passoni et al., 2002). The PBLs were restimulated and supplemented with 20 IU IL-2 ml−1 24 h later. PBLs were restimulated each week in the same manner. Seven days after the fourth round of restimulation, cells were harvested and isolated from the bulk culture by CD4+ cell-negative depletion using mAb-coated beads (Dynal Dynabeads). Then, the resulting cells were tested by ELISPOT assays and cytotoxicity assays.

IFN-γ ELISPOT assays were performed by using a commercially available kit (Diacclone). Resulting spots were counted by using an ImmunoSpot analyser (Cellular Technology Ltd) and scored as spot-forming cells (SFC). A standard 4 h 51Cr-release assay was used to measure CTL activity as described above. A 10-fold excess of unlabelled K562 cells was added to offset natural killer activity. Spontaneous and total releases of 51Cr were calculated by incubating target cells in each experiment with medium and with 2% Triton X-100, respectively. The percentage of specific lysis was calculated by the following formula: specific lysis (%) = 100 × (mean experimental c.p.m. – mean spontaneous c.p.m.)/(mean maximum c.p.m. of total release – mean spontaneous c.p.m.).

For each peptide, PBL samples from four donors were tested. Three of the four healthy HLA-A*0201+ individuals were responders to stimulation with P340–348. The CTLs from the three donors produced IFN-γ in a dose-dependent manner when incubated with P340–348-loaded T2 cells and lysed P340–348-loaded T2 cells, but did not respond to either the irrelevant peptide HBCag131–140-loaded T2 cells or T2 cells alone (Fig. 1a, b). The CTL activity of the peptide P340–348-induced effectors could be inhibited significantly by the anti-HLA-A2 antibody, consistent with a class I-restricted mechanism of cytotoxicity (Fig. 1b). No specific reactivity was detected in the cells generated from PBLs stimulated with P333–341 (data not shown).

To address whether the immunogenic epitope P340–348 could be processed and presented naturally, 100 ± 20 m.o.i. human RV Wa strain-infected Caco-2 (HLA-A*0201+) and HT-29 (HLA-A*0201+) cells were used as target cells (Offit et al., 1983). The CTLs that lysed peptide-loaded T2 cells specifically were capable of killing human RV Wa strain-infected Caco-2 cells in an HLA-A2-restricted manner. No lysis of Caco-2 cells, HT-29 cells or human RV Wa strain-infected HT-29 cells was observed (Fig. 1c). These results indicated that P340–348 could be processed and presented naturally in the context of HLA-A*0201 molecules on a human colorectal adenocarcinoma cell line.
HLA-A*0201/Kb transgenic mice, between 6 and 8 weeks old (Jackson Laboratory) were used to determine the P340–348-specific CTL response by intraperitoneal administration of 10⁷ p.f.u. cell-cultured human RV Wa strain. The transgenic mice were sacrificed by vertebral dislocation 6 days after immunization in accordance with local ethical guidelines. Single-cell suspensions of these mouse spleens were prepared by needle dissection and filtered through a 100-mesh sieve. Stimulator cells (splenocytes obtained from non-immune syngeneic mice) were incubated with 50 μM P340–348 for 3 h and then irradiated at 2000 rad. Then, the stimulator cells were co-cultured with the effector-cell population in a 1:2 ratio. On day 5, the induced cells were assessed by an ELISPOT assay and a standard 4 h ⁵¹Cr-release assay. The cells showed IFN-γ production to P340–348-loaded T2 cells in a dose-dependent manner and lysed P340–348-loaded T2 cells, but did not respond to either the irrelevant peptide HBcAg131–140-loaded T2 cells or T2 cells alone (Fig. 2a, b). These results indicated that P340–348 was also immunogenic in HLA-A*0201/Kb transgenic mice.

In conclusion, this study has identified an HLA-A*0201-restricted CTL epitope, P340–348 (TLLANVTAV), derived from the human RV Wa strain VP6 protein. This epitope had a high affinity for HLA-A*0201 molecules, predicted by computer algorithms and verified by a major histocompatibility complex peptide-binding assay. This epitope was further examined by inducing specific CTL responses in vitro from HLA-A*0201-matched healthy-donor PBLs and in vivo in HLA-A*0201/Kb transgenic mice. CTLs elicited by P340–348 stimulation secreted IFN-γ and killed target cells in an antigen-specific and HLA-A*0201-restricted manner. Furthermore, P340–348 could be processed endogenously and presented in the context of HLA-A*0201 molecules on the surface of a human colorectal adenocarcinoma cell line. To the best of our knowledge, this study is the first successful identification of a novel HLA-A*0201-restricted CD8+ T-cell epitope from RV proteins. We suggest that this newly identified epitope will help in the characterization of RV-protection mechanisms in humans and may be relevant to the development of vaccine design/evaluation approaches for RV.

**Fig. 1.** Specific reactivity of human CTLs induced by autologous P340–348-loaded DCs in vitro. (a) Number of IFN-γ SFC per million CD8+ T cells stimulated by T2 cells loaded with the indicated concentrations of P340–348, irrelevant peptide HBcAg131–140 and medium in an ELISPOT assay. (b, c) Specific lysis (%) of the CTLs against the indicated target cells in a standard 4 h ⁵¹Cr-release assay. (a) CTLs released IFN-γ in response to P340–348-loaded T2 cells in a dose-dependent manner, but not in response to irrelevant peptide HBcAg131–140-loaded T2 cells or T2 cells alone. (b) CTLs efficiently recognized and killed P340–348-loaded T2 cells in an HLA-A2-restricted way. (c) Caco-2 cells (HLA-A*0201+) and HT-29 cells (HLA-A*0201 −) were infected with 100 ± 20 m.o.i. of human RV Wa strain and then used as target cells. Results of one representative experiment of at least three performed are shown.

**Fig. 2.** Immunogenicity of P340–348 in HLA-A*0201/Kb transgenic mice. (a) T2 cells were incubated with the indicated concentrations of P340–348 and HBcAg131–140 and were then used as stimulator in an ELISPOT assay. (b) The bulk CTLs lysed P340–348-loaded T2 cells efficiently and no activity was detected against HBcAg131–140-loaded T2 cells or T2 cells alone. Results of one representative experiment of at least three performed are shown.
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References


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