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Identification of *Streptococcus mutans* PAc peptide motif binding with human MHC class II molecules (*DRB1*0802*, **1101*, **1401* and **1405*)

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SUMMARY

A surface protein antigen (PAc) of *Streptococcus mutans*, in particular the A-region of this PAc molecule, has been noted as a possible target in research for an effective dental caries vaccine. To identify the antigenic peptide binding to major histocompatibility complex (MHC) class II (HLA-DR) molecules in the A-region, we prepared a panel of overlapping synthetic peptides in the second unit of the A-region, and established that a simple enzyme-linked immunosorbent assay (ELISA) binding assay could be achieved by incubating the DR-crude. Binding to DR molecules of these peptides from nine donors was investigated by using the ELISA binding assay. It was revealed that the PAc(316–334) peptide bound more strongly to the HLA-DR molecule in seven out of nine subjects. In particular, DR8 (*DRB1*0802*), DR5 (*DRB1*1101*) and DR6 (*DRB1*1402* and **1405*), which bound strongly to PAc(316–334) peptide, were identified. Moreover, we synthesized glycine-substituted peptide analogues of the peptide and examined the binding motif of the binding region. As a result, the multiple binding motif in DR8, DR5 and DR6 was found in L-RV-K-A. It is suggested that a peptide vaccine for dental caries that is more effective for humans, with fewer adverse side-effects, could be designed by combining the multiple binding motif with the B-cell epitope to produce only the inhibiting antibody against dental caries. The peptide could therefore be useful for peptide vaccine development in the general human population.

INTRODUCTION

Streptococcus mutans has attracted wide attention over the years as a major pathogen in the aetiology of dental caries. A cell-surface protein antigen of *S. mutans*, which has been called PAc,[1] AgI/II,[2,3] PI,[4] B[5] and MSL-1,[6] functions essentially by colonizing the tooth surface, and interacts with the salivary pellicle that coats the dental enamel.[7–9] The past several years have witnessed an increasing interest in the study of a vaccine for dental caries using this protein.[10–12] Recently, a peptide vaccine making use of this protein has been studied and may be a safe vaccine for dental caries because dental caries is not a morbid disease. Different groups have reported various peptide antigens containing strongly immunogenic and essential epitopes.[13–15] In general, the advantage of a peptide antigen is that peptides are high in chemical purity, stable for a long period of time, and easy to preserve; exclusion of unnecessary and inconvenient epitopes can be avoided as antigen epitopes. Furthermore, they can be made to conform to a minimum unit, and a desired antigen can be designed freely. As for disadvantages, peptides exhibit weak immunogenicity.[16,17]

Many methodological variations using B- and T-cell epitopes have been developed for a synthetic peptide vaccine to enhance the low immunogenicity of the peptides without protein carriers.[18–20] Recently, in the PAc molecule, it was reported that PAc(301–319)[13] and PAc(361–377)[21] peptides in the alanine-rich repeating region (residues 219–464, A-region) possessed the immunodominant T- and B-cell epitope and gave strong antigenicities of these peptides. Some peptides, which possessed the immunodominant and minor T- and B-cell epitopes, were also identified within residues 806–853, 1005–1054 and 1085–1134 in the prolin-rich repeating region (residues 816–1213, P-region).[15] In particular, PAc(361–377) peptide, including the PAc(residues 365–377, 365–377) in which T- and B-cell epitopes overlap, could produce significant amounts of antibodies, most of which have exhibited a cross-reaction with PAc. In addition, it was revealed that the antibody inhibited $\approx 50\%$ of the binding of fluid-phase salivary components to immunized recombinant (r)PAc in the BIAcore instrument (optical biosensors).[14] It was also shown that PAc(305–318) peptide coupled to the N-terminal region of PAc(361–377) peptide elevated cross-reacting antibody levels, compared to immunization with PAc(361–377) peptide only, in mice of H-2^f, H-2^d, H-2^a, H-2^k

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and H-2^s.[21] These reports suggested to us a feasible strategy to design some peptide-based vaccines using T- and B-cell epitopes for *S. mutans*. However, the practical analysis of the immune response in humans is indispensable for the development of any vaccine antigen. The human major histocompatibility complex (MHC) encodes at least three polymorphic series of HLA class II antigens, designated HLA-DR, -DQ and -DP, which are highly polymorphic cell-surface proteins consisting of α and β chains. HLA class II molecules are constructionally expressed on the cell surface of B cells, monocytes and dendritic cells in the peripheral lymphoid organs, and on epithelial and dendritic cells in the thymus. The HLA class II molecules have the function of presenting antigenic peptides to $CD4^+$ T cells. Therefore, to make synthetic peptide vaccines effective against dental caries, one would have to incorporate enough antigenic determinant to mediate binding by most HLA types.

In this study, we synthesized a set of 19-mer peptides in the second unit of the A-region of *S. mutans* PAc and established an enzyme-linked immunosorbent assay (ELISA) binding assay to identify the DR-binding region. We attempted to identify PAc DR-binding peptides in order to develop potent immunogens for MHC class II-restricted T cells by the ELISA binding assay. Although not all of the peptides were as widely recognized as expected, the PAc(316–334) peptide was identified and was broadly recognized by several HLA-DR types. Furthermore, the peptide-binding region of several DR epitopes was identified by truncation analysis, and a putative DR-binding motif has been proposed.

MATERIALS AND METHODS

Antibodies

L243 anti-DR αβ[22] was purified from hybridoma culture supernatant using protein A–Sepharose beads (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden).

B-lymphoblastoid cell line (B-LCL)

Human monocytes were prepared from the peripheral blood of healthy donors between 22 and 45 years of age and immortalized with the B95-8 cell line supernatant according to Bird *et al.*[23] Epstein–Barr virus (EBV)-infected lymphocytes were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) for 15 days, and the immortalized cells, B-LCL, were then obtained. The B-LCL were used as sources of human HLA class II molecules: SAA; DR8 (*DRB1* 0802*), HSA; DR6/2 (*DRB1*1401/*1502*), NUA; DR5/2 (*DRB1*1101/*1501*), TIA; DR9/2 (*DRB1* 0901/*1501*), KTA; DR4/6 (*DRB1*0406/*1302*), MIA; DR6/2 (*DRB1*1405/*1501*), AMO; DR8/9 (*DRB1*08032/*0901*), MMA; DR4/6 (*DRB1*0405/*1405*), HKA; DR1/2 (*DRB1*0101/*1502*). The genetic (phenotypic) typing for HLA-DR was performed in the Tissue Typing Department (BML, Tokyo, Japan).

Preparation of crude HLA-DR molecules

The preparation of crude HLA-DR molecules was performed as described previously.[24] Approximately 5×10^9 cells were lysed in 0·15 M NaCl–0·01 M Tris (TS) (pH 7·4), containing 2% polyoxyethylene-9-lauryl ether ($C_{12}E_9$), 5 mM iodoacetamide and the proteinase inhibitors N-tosyl-L-lysine chloromethyl ketone (100 μM) and phenylmethylsulphonyl fluoride (PMSF) (500 μM), for 60 min on ice. Nuclear material was removed from the cell lysates by centrifugation at 500 ***g*** for 10 min at 4°. The cell extract was further clarified by ultracentrifugation at 100 000 ***g*** for 1 hr at 4°. The pellets were suspended in TS containing 0·1% $C_{12}E_9$ and stored frozen until used.

Synthetic peptides

All PAc peptides are listed in Table 1. These peptides were all derived from the sequence of the PAc gene from *S. mutans* MT8148, which corresponds to part of the alanine-rich repeat as already described by Okahashi *et al.*[25] The peptides were synthesized by a step-wise solid-phase procedure using a Model 350 Multiple Peptide Synthesizer (Advanced Chemtech, Louisville, KY).[26,27] Side-chain-protected peptides on resin were biotinylated with excess N-hydroxysuccinimido-biotin (Sigma Chemical Co., St Louis, MO). They were subsequently purified by reverse-phase high-performance liquid chromatography (HPLC) on a TSK-GEL column (1 × 30 cm) (Tosoh, Tokyo, Japan), with a 10–45% acetonitrile gradient in 0·1% trifluoroacetic acid (TFA) developed over 50 min at a flow rate of 5 ml/min. The purity of these peptides was > 95% as determined by HPLC analysis. To confirm the amino acid sequence of the synthetic peptides, several peptides were selected by random sampling and further analysed by using a System 7300 Amino Acid Analyzer (Beckman Instruments, Fullerton, CA) and a Model 477 A Protein Sequencer (Applied Biosystems, Foster City, CA).

Binding assay

Ninety-six-well microtitre H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4° with 100 μl of 10 μg/ml L243 monoclonal antibody in 50 mM carbonate buffer, pH 9·6. The plates were washed with phosphate-buffered saline (PBS) containing 0·1% (v/v) Tween-20 (PBST) and blocked with 1% (w/v) skimmed milk in PBST for 1 hr at 37°. Biotinylated peptide in PBS was added to the B-LCL extracts in 0·1 M citrate/phosphate buffer (pH 5·0) containing 0·1% $C_{12}E_9$ and preincubated at 37° overnight. The reaction mixtures were added to the L243 monoclonal antibody-coated ELISA plate, from which the excess skimmed milk was removed by three washes with PBST, and incubated for 1 hr at 37°. The wells were then washed five times with PBST and further incubated for 1 hr at 37° with 100 μl of alkaline phosphatase-conjugated streptavidin. After five washes with PBST, bound complexes between the HLA-DR molecule and biotin–PAc peptides were detected by the addition of 100 μl of 1 mg/ml paranitrophenyl phosphate as a substrate, and incubated for 1 hr at 37°. The absorbance at 405 nm (A_{405}) was measured with a microplate reader (MPR A4; Tosoh). In the inhibition assay for the binding of the HLA-DR molecule and biotin–PAc peptides, the B-LCL extracts were preincubated with 1 μg biotin–PAc peptide and various concentrations of non-biotinylated PAc peptide for 1 hr at 37°. The reaction mixtures were added to the L243 monoclonal antibody-coated ELISA plate (100 μl/well) and incubated for 1 hr at 37°. The bound antibodies were detected as described above.

Human peripheral blood mononuclear cells (PBMC) proliferation assay

Human mononuclear cells were prepared from peripheral blood and adjusted to a concentration of 1×10^7 cells/ml in

Table 1. Amino acid sequences of overlapping synthetic peptides covering the second unit of the A-region and the glycine substitute of PAc(316–331) and PAc(316–334)

Peptide	Position†	Amino acid sequence‡
PAc(301–319)	301–319	ANAANEADYQAKLTA YQTE
PAc(316–334)	316–334	YQTE LARVQKANADA KATY
PAc(331–349)	331–349	KATY EAAVAANNAKN AALT
PAc(346–364)	346–364	AALT AENTAIKQRNE NAKA
PAc(361–379)	361–379	NAKA TYEAALKQYEA DLAA
PAc(376–394)	376–394	DLAA VKKANAANEADTQAK
PAc(308–326)*	308–326	DYQAKLTAYQTELARVQKA
PAc(325–343)*	325–343	KANADAKATYEAAVAANNA
PAc(338–356)*	338–356	VAANNAKNAALTAENTAIK
PAc(355–373)*	355–373	IKQRNENAKATYEAALKQY
PAc(369–387)*	369–387	AALKQYEADLAAVKKANAA
PAc(316–331)	316–331	YQTELARVQKANADAK
PAc(316–331), 1G§	316(G)–331	GQTELARVQKANADAK
PAc(316–331), 2G	316–317(G)–331	YGTELARVQKANADAK
PAc(316–331), 3G	316–318(G)–331	YQGELARVQKANADAK
PAc(316–331), 4G	316–319(G)–331	YQTGLARVQKANADAK
PAc(316–331), 5G	316–320(G)–331	YQTEGARVQLANADAK
PAc(316–331), 6G	316–321(G)–331	YQTELGRVQKANADAK
PAc(316–331), 7G	316–322(G)–331	YQTELAGVQKANADAK
PAc(316–331), 8G	316–323(G)–331	YQTELARGQKANADAK
PAc(316–331), 9G	316–324(G)–331	YQTELARVGKANADAK
PAc(316–331), 10G	316–325(G)–331	YQTELARVQGANADAK
PAc(316–331), 11G	316–326(G)–331	YQTELARVQKGNADAK
PAc(316–331), 12G	316–327(G)–331	YQTELARVQKAGADAK
PAc(316–331), 13G	316–328(G)–331	YQTELARVQKANGDAK
PAc(316–331), 14G	316–329(G)–331	YQTELARVQKANAGAK
PAc(316–331), 15G	316–330(G)–331	YQTELARVQKANADGK
PAc(316–331), 16G	316–331(G)	YQTELARVQKANADAG
PAc(316–334), 1G	316(G)–334	GQTELARVQKANADAKATY
PAc(316–334), 2G	316–317(G)–334	TGTELARVQKANADAKATY
PAc(316–334), 3G	316–318(G)–334	YQGELARVQKANADAKATY
PAc(316–334), 4G	316–319(G)–334	YQTGLARVQKANADAKATY
PAc(316–334), 5G	316–320(G)–334	YQTEGARVQKANADAKATY
PAc(316–334), 6G	316–321(G)–334	YQTELGRVQKANADAKATY
PAc(316–334), 7G	316–322(G)–334	YQGELAGVQKANADAKATY
PAc(316–334), 8G	316–323(G)–334	YQTELARGQKANADAKATY
PAc(316–334), 9G	316–324(G)–334	YQTELARVGKANADAKATY
PAc(316–334), 10G	316–325(G)–334	YQTELARVQGANADAKATY
PAc(316–334), 11G	316–326(G)–334	YQTELARVQKGNADAKATY
PAc(316–334), 13G	316–328(G)–334	YQTELARVQKANGDAKATY
PAc(316–334), 14G	316–329(G)–334	YQTELARVQKANAGAKATY
PAc(316–334), 15G	316–330(G)–334	YQTELARVQKANADGKATY
PAc(316–334), 16G	316–330(G)–334	YQTELARVQKANADAGATY
PAc(316–334), 17G	316–330(G)–334	YQTELARVQKANADAKGTY
PAc(316–334), 18G	316–330(G)–334	YQTELARVQKANADAKAGY
PAc(316–334), 19G	316–334(G)	YQTELARVQKANADAKATG

†Six sequential overlapping (four amino acid residues) 19-mer peptides covering second units of the A-region in the PAc molecules and five 19-mer peptides (*) containing the above overlapping four amino acid residues at the middle of the molecules were designed according to Okahaski *et al.*[25]

‡PAc(316–331), 1G indicated the PAc(316–331) peptide with a glycine for tyrosine at position 316.

§The sequences are given in one-letter code.

RPMI-1640 medium supplemented with 2 mM L-glutamine (Gibco), 15 mM N-2-hydroxyethylpiperazine-N′-2-etane-sulphonic acid (HEPES) buffer, 20 mM sodium bicarbonate (Wako Pure Chemical Industries Ltd, Osaka, Japan), 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercapto-ethanol and 10% heat-inactivated FCS. One-hundred microlitres of the human monocyte fraction (1×10^6 cells/well) were cultured with 100 μl of the above medium containing the test peptide (5 μg/well) under a humidified atmosphere of 5% CO_2 in air for 3 days and pulsed for a final 6-hr cultivation with 1 μCi of [^{3}H]thymidine per well (ICN Radiochemicals, Irvine, CA), with the cells being finally harvested onto glass fibres

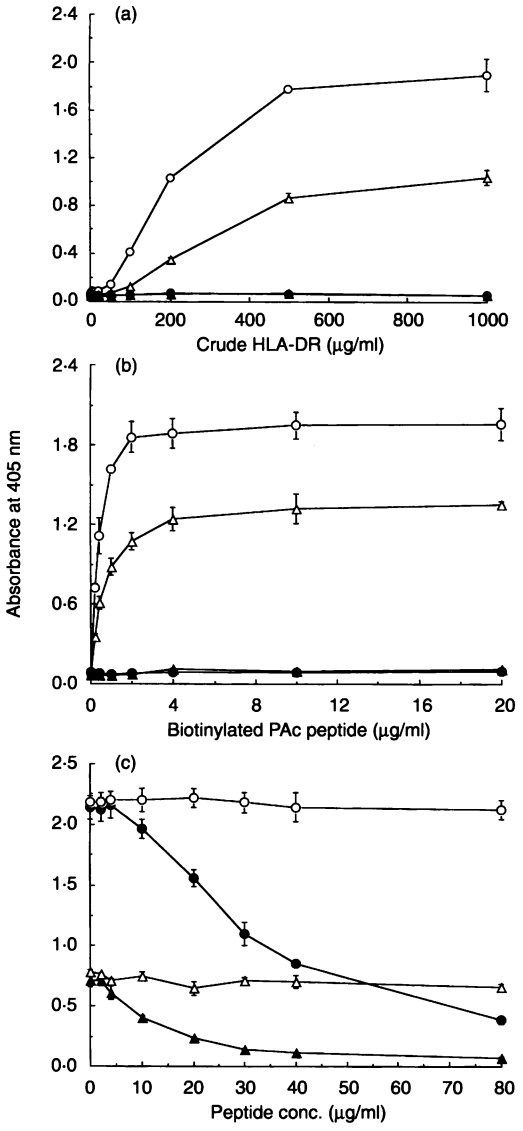

Figure 1. Binding responses of synthetic peptides to HLA-DR molecules. (a) Binding of PAc(316–334) and PAc(346–364) peptides to various HLA-DR concentrations. Biotinylated PAc(316–334) peptide at a concentration of 5 μg/ml was incubated with various concentrations of SAA DR8 (*DRB1*0802*) (○) and HSA DR6/2 (*DRB1*1401/*1502*) (△). Biotinylated PAc(346–364) peptide 5 μg/ml was also incubated with various concentrations of SAA DR8 (*DRB1*0802*) (●) and HSA DR6/2 (*DRB1*1401/*1502*) (▲). At the end of the experiment, samples were analysed by plate assay as described in the Material and Methods. The results are shown as mean absorbance at 405 nm (A_{405}) ± SD for triplicate assays. The experiments were performed three times, and similar results were obtained in each one. (b) Quantification of biotinylated PAc(316–334) (○) and biotinylated PAc(346–364) (●) peptides bound to SAA DR8, and biotinylated PAc(316–334) (△) and biotinylated PAc(346–364) (▲) peptide bound to HSA DR6/2 at various peptide concentrations. (c) The competitive binding responses of biotinylated PAc(316–334) peptide in the presence of either PAc(316–334) or PAc(346–364). Crude SAA DR8 at a concentration of 500 μg/ml was incubated with biotinylated PAc(316–334) 2 μg/ml and in the presence of 0–80 μg/ml of either PAc(316–334) (●) or PAc(346–364) (○) peptide at 37° overnight. Crude HSA DR6/2 was also incubated with biotinylated PAc(316–334) in the presence of either PAc(316–334) (▲) or PAc(346–364) (△) under the same assay conditions. The amounts of biotinylated PAc(316–334) peptide associated with HLA-DR 8 and DR 6/2 were then quantified as described in the Materials and Methods. The results were shown as mean absorbance at 405 nm (A_{405})±SD for triplicate assays. The experiments were performed three times, and similar results were obtained in each one.

using a multiple-cell harvester. The incorporated radioactivities into proliferating cells were determined with a liquid scintillation counter. The proliferative responses were expressed as the mean counts per minute (c.p.m.) incorporated, or a stimulation index (SI) that was the ratio of mean c.p.m. incorporated in the presence of antigen to those obtained in the absence of antigen.

RESULTS

Proliferative response to synthetic peptides of peripheral blood lymphocytes

Serum samples from the two donors, SAA (*DRB1*0802*) and HSA (*DRB1*1401/*1502*) in whom *S. mutans* was isolated, reacted with *S. mutans* PAc (data not shown). Therefore, it is believed that the polyclonal T cells from the two donors reacted with some antigenic PAc peptides. To establish a binding assay between HLA-DR molecules and PAc peptides, as a primary experiment, the reactivities of the peripheral T cells to PAc peptides were tested in the two donors. A set of sequential overlapping 19-mer peptides covering the second unit in the A-region of PAc molecules, which interact with salivary components, was used in the peripheral T-cell proliferation assay. The PBMC from the two donors showed proliferative responses to PAc(316–334) peptide (SI > 2·5) but not to other peptides (data not shown). Therefore, it is very likely that PAc(316–334) peptide possessed a T-cell epitope and DR-binding motif in DR8 (*DRB1*0802*) and DR6/2 (*DRB1*1401/*1502*).

Binding characteristics of PAc(316–334) and PAc(346–364) peptide with HLA-DR molecules

To identify the DR-binding region in PAc molecules, we prepared DR-crude samples from two kinds of B-LCL, DR8 (*DRB1*0802*) and DR6/2 (*DRB1*1401/*1502*). To establish the DR-binding ELISA between the synthetic peptides and DR molecules, we tested the reactivities of PAc(316–334) peptide with various HLA-DR concentrations in the ELISA binding assay (Fig. 1a). PAc(346–364) peptide, which did not proliferate PBMC, was used as a negative control in the DR-binding assay. As a result, the reactivity of PAc(316–334) peptide to HLA-DR 8 and DR 6/2 molecules rose in a dose-dependent manner according to DR concentration, and almost reached the plateau observed at 500 μg/ml (Fig. 1a). HLA-DR8 and DR6 (2500 μg/ml) were mixed with various PAc peptide concentrations. The reactivities of PAc(316–334) peptide to SAA DR8 and HSA DR6/2 rose immediately, and almost reached the plateau to react with the PAc peptide, 2 μg/ml after mixing (Fig. 1b). However, PAc(346–364) peptide did not show binding responses to the two types of HLA-DR (Fig. 1a,b). The crude samples from spleen cells in B10.D2 mice, which were used as HLA-DR non-expressing

cells, did not show positive response to PAc(316–334) and PAc(346–364) peptide in this assay (data not shown).

To confirm the specificity of the binding, the inhibitory effects of non-labelled PAc peptides on the binding of biotin-labelled PAc peptides to two HLA-DR molecules were examined by the ELISA competitive inhibition assay (Fig. 1c). The ELISA responses were inhibited in a dose-dependent manner and, according to DR concentration, by non-labelled PAc(316–334) peptide. The inhibitory effect reached a maximum at a peptide concentration of 80 μg/ml. The significant inhibitory effects were not observed for PAc(346–364) peptide, even with elevation in the concentration. From the above results, it was clear that PAc(316–334) peptide bound specifically to HLA-DR8 and DR6/2 in the binding assay, thus establishing the assay conditions.

Identification of a PAc-binding motif to the HLA-DR8, DR11 and DR6

The second unit in the A-region of the PAc molecule is a strong antigenic region in both the murine[28,29] and human[30,31] immune system. We therefore synthesized six overlapping 19-mer peptides covering the second unit in the A-region of the PAc molecules, and five 19-mer peptides containing the same four overlapping amino acid residues in the middle of the molecules. To identify the PAc peptide-binding region to various HLA-DR molecules, we examined the reactivities of these PAc peptides to the HLA-DR molecule samples from nine donors in the ELISA binding assay. The results are shown in Table 2. PAc(316–334) peptide bound strongly to HLA-DR molecules from seven out of nine donors. PAc(369–387), PAc(361–379) and PAc(325–343) peptides strongly bound to HLA-DR molecules in four, three and three, respectively, out of nine subjects. PAc(301–319) peptide bound significantly to two types of HLA-DR molecule. Among the HLA-DR molecules that bound strongly to PAc(316–334) peptide, the HLA-DR genotype from SAA was homozygous (*DRB1 *0802*) but others were heterozygous (NUA, *DRB1*1101/*1501*; HSA, *DRB1*1401/*1502*; MIA, *DRB1*1405/*1501*; KTA, *DRB1*0406/*1302*; MMA, *DRB1*0405/*1405*; AMO, *DRB1*08032/*0901*). The HLA-DR molecules of TIA that bound weakly to PAc(316–334) peptide were TIA DR9/2 (*DRB1*0901/*1501*) and HKA DR1/2 (*DRB1*0101/1502*). Because DR1 (*DRB1*0101*), DR9 (*DRB1*0901*), DR2 (*DRB1*1501*) and DR2 (*DRB1*1502*) bound weakly to PAc(316–334) peptide, it was suggested that DR5 (*DRB1*1101*), DR6 (*DRB1*1401* and **1405*) and DR8 (*DRB1*08032*) would bind strongly to PAc(316–334) in NUA, HSA, MIA and AMO. The high reactivity of the HLA-DR molecule to PAc(316–334) peptide in MMA, with DR molecule type DR4/6 (*DRB1*0405/*1405*), depended on DR6 (*DRB1*1405*). The DR type that bound to the PAc(316–334) peptide in KTA DR4/6 (*DRB1*0406/*1302*) was not identified.

In order to design a multiple peptide vaccine for dental caries in humans, we attempted to identify the precise DR-binding region in the second unit of the A-region of the PAc molecule. We synthesized 14 truncated PAc(316–334) peptides: two 18-mer peptides, each sequentially overlapping the previous one by 17 amino acids; three 17-mer peptides, each sequentially overlapping the previous one by 16 amino acids; four 16-mer peptides, each sequentially overlapping the previous one by 15 amino acids; five 15-mer peptides, each sequentially overlapping the previous one by 14 amino acids. As a result, the precise DR-binding region on the PAc(316–334) peptide was identified by ELISA binding assay using these truncated peptides in SAA, NUA, HSA and MIA. PAc(316–331) peptide, which lacked three (Gln, Thr and Glu) residues at the C-terminal end of the PAc(316–334) peptide, bound to the HLA-DR molecules from SAA and HSA. The binding activity of PAc(316–331) peptide was to the same degree as that of PAc(316–334) peptide. However, in NUA and MIA the binding activities of these truncated PAc(316–334) peptides were weak in comparison to that of PAc(316–334) peptide (data not shown).

We also attempted to define the crucial DR contact residues as good DR binders, such as PAc(316–331) peptide in SAA and HSA, and PAc(316–334) peptide in NUA and MIA. The biotinylated 16 single amino acid substitutions of PAc(316–331) and 19 single amino acid substitutions of PAc(316–334) peptide were investigated for their capacity to bind DR8 (SAA), DR6 (HSA), DR5 (NUA) and DR6 (MIA) molecules by using the ELISA binding assay. The results are shown in Fig. 2 and the data summarized in Table 3. In SAA, of the 16 possible single glycine-substituted analogues of PAc(316–331) only three, PAc(316–331) 5G, 8G and 10G, did not bind to the HLA-DR8 molecule (Fig. 2a). Therefore, three residues (L320, V323 and K325) appear to be the crucial DR8 contact residues. In a similar assay, it also appeared that four residues (L320, R322, V323 and K325) were the crucial DR5 contact residues (Fig. 2c). Five residues (L320, R322, V323, K325 and A328) that appeared to be crucial for DR6 (*DRB1*1401*) binding also appeared to play an important role for DR6 (*DRB1*1405*) PAc(316–334) peptide interactions (Fig. 2b,d).

DISCUSSION

In the present report, we have described the establishment of binding assays for HLA-DR molecules by using crude MHC and biotinylated peptides. We modified a previously described assay condition[24,31] for incubation between the biotinylated peptides and the crude DR samples. Purified MHC class II binds peptide extremely slowly, requiring 24–48 hr to reach saturation.[32,33] By contrast, it is reported that biochemical peptide binding to MHC class II molecules occurs rapidly under a cofactor, such as when intracellular HLA-DM is present.[34] The peptide association rate can be strikingly enhanced at the low pH characteristic of endsomal components.[35,36] To establish the binding assay under stable conditions, the crude HLA-DR samples, including some cofactor in intracellular and outer membranes, were incubated with biotinylated peptides at pH 5·0, 37°, overnight. The peptide–DR complexes were estimated by using an anti-HLA-DR monoclonal antibody (L243)-coated plate and quantified by colorimetric method using alkaline phosphatase-coupled streptavidin. Our data confirmed a positive association between the peptide by which the peripheral T cells were stimulated and two different DR molecules, without purification of HLA-DR molecules (Fig. 1).

In designing an effective vaccine for humans, multiple T-cell epitopes should be contained in peptide antigens in order to respond to the many types of HLA-DR, as humans

Table 2. Identification of agretopes in the A2 unit of the A-repeat

			PAc peptide										
Donor	DRB1	None	(301–319)	(316–334)	(331–349)	(346–364)	(361–379)	(376–394)	(308–326)	(325–343)	(338–356)	(355–373)	(369–387)
SAA	0802/0802	0·072±0·003*	0·668±0·046	1·650±0·034	0·133±0·008	0·079±0·002	0·271±0·038	0·080±0·004	0·148±0·015	0·144±0·014	0·112±0·049	0·190±0·005	1·330±0·019
NUA	1101/1501	0·104±0·076	0·503±0·076	2·097±0·067	0·338±0·016	0·131±0·030	0·646±0·076	0·104±0·004	0·488±0·031	0·337±0·037	0·211±0·005	0·313±0·001	1·235±0·019
HSA	1401/1502	0·074±0·002	0·088±0·002	0·693±0·016	0·156±0·003	0·087±0·001	0·104±0·009	0·078±0·002	0·258±0·003	0·227±0·008	0·173±0·024	0·088±0·002	0·683±0·029
TIA	0901/1501	0·091±0·015	0·207±0·034	0·263±0·034	0·401±0·027	0·165±0·008	0·747±0·040	0·167±0·011	0·264±0·031	1·153±0·061	0·160±0·023	0·263±0·048	0·208±0·028
KTA	0406/1302	0·127±0·010	0·134±0·010	0·507±0·013	0·505±0·026	0·222±0·006	0·178±0·017	0·180±0·011	0·147±0·016	0·146±0·003	0·189±0·007	0·135±0·005	0·189±0·007
MIA	1405/1501	0·067±0·005	0·071±0·001	0·100±0·032	0·321±0·018	0·081±0·003	0·204±0·015	0·075±0·004	0·322±0·009	0·274±0·016	0·084±0·003	0·085±0·014	0·454±0·033
AMO	08032/0901	0·101±0·008	0·398±0·016	1·020±0·050	0·313±0·067	0·126±0·001	0·845±0·021	0·252±0·013	0·182±0·010	1·690±0·061	0·327±0·049	0·720±0·032	0·479±0·032
MMA	0405/1405	0·046±0·001	0·053±0·003	1·000±0·049	0·212±0·008	0·066±0·001	0·097±0·001	0·064±0·001	0·092±0·001	0·469±0·007	0·046±0·001	0·057±0·001	0·415±0·007
HKA	0101/1502	0·070±0·005	0·101±0·001	0·228±0·007	0·449±0·014	0·078±0·001	0·210±0·003	0·076±0·001	0·442±0·014	1·130±0·036	0·163±0·071	0·089±0·003	0·530±0·028

*This range is absorbance at A_{405}.

Biotinylated PAc peptides at a concentration of 5 μg/ml were incubated with various HLA-DR molecules at a concentration of 500 μg/ml. At the end of the experiment, samples were analysed by plate assay as described in the Materials and Methods. The results are shown as mean absorbance at 405 nm (A_{405})±SD for triplicate assays. The experiments were performed three times, and similar results were obtained in each one.

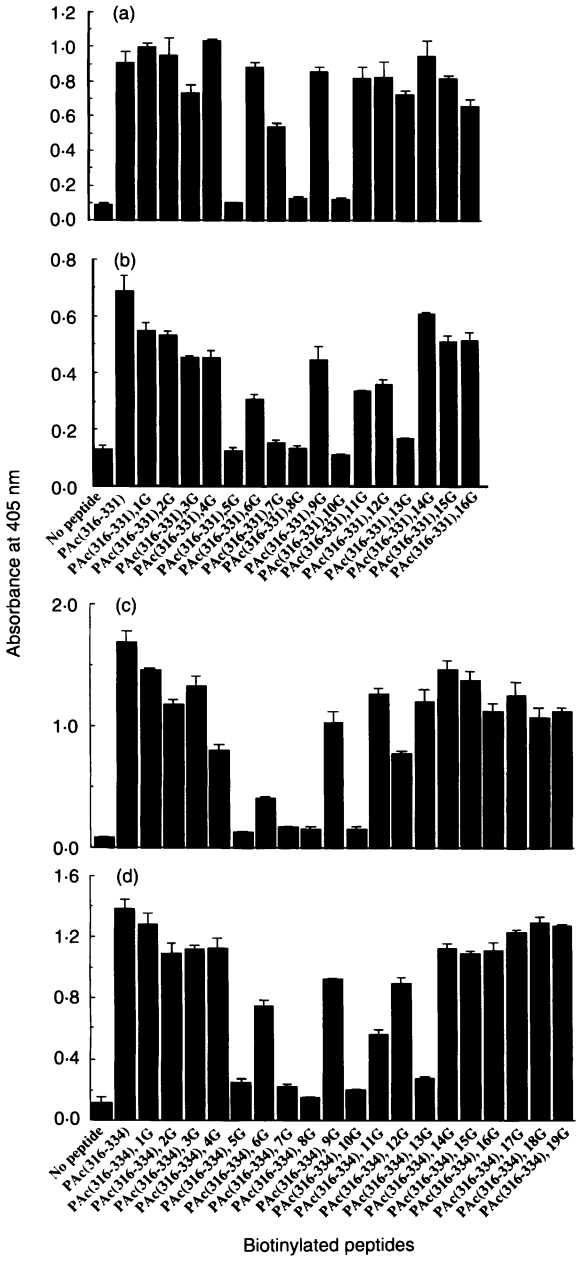

Figure 2. Binding responses of PAc(316–331) and PAc(316–334) analogue peptides to HLA-DR molecules. Biotinylated PAc(316–331) analogue peptides at a concentration of 5 μg/ml were incubated with SAA DR8 (a) and HSA DR6/2 (b) at a concentration of 500 μg/ml. Biotinylated PAc(316–334) analogue peptides at a concentration of 5 μg/ml were incubated with NUA DR5/2 (c) and MIA DR6/2 (d) at a concentration of 500 μg/ml. Analogue peptides were designated by the amino acid substituted for the wild-type residue at that position. PAc(316–331), 1G indicates the PAc(316–331) peptide with a glycine substituted for the tyrosine at the position 316. At the end of the experiment, samples were analysed by plate assay as described in the Material and Methods. The results are shown as mean absorbance at 405 nm (A_{405}) ± SD for triplicate assays. The experiments were performed three times, and similar results were obtained in each one.

demonstrate a variety of HLA types. In general, T-cell epitopes are deeply involved in binding with MHC molecules and antigen presentation to T cells, mediated by antigen-presenting cells.[37,38] Each T-cell epitope at first binds with MHC molecules, then it is recognized by the T cell. Therefore, the binding between a MHC molecule and peptide is important.[39] The antigenic determinant that can bind to a MHC molecule differs, depending on each MHC molecule. Meanwhile, the affinity between a MHC molecule and a peptide affects the reactivity of the peptide with the MHC-bound complex and a T cell.[39] For preparing a more effective peptide vaccine, it is important to uncover a peptide with a high affinity for MHC.

In the present study, we attempted to identify PAc DR-binding peptides to develop potent immunogens for MHC class II (HLA-DR)-restricted T cells by the ELISA binding assay. Because of the extensive information that has been accumulated in our laboratory and elsewhere on the detailed structure/function analysis of the A-region in the PAc molecule, with respect to the contribution of different regions within this A-region to T- and B-cell recognition, we used the second unit in the A-region as a starting point to construct the PAc DR-binding peptide. As a result, it was revealed that PAc(316–334) peptide bound more strongly to the HLA-DR molecule in seven out of nine subjects (Table 2). It has been reported that the PAc(316–334) peptide possessed dominant B-cell epitopes in an analysis using serum after hu-PBL-SCID mice were immunized with PAc.[40] Moreover, it has been proven that PAc(316–334) peptide contains B-cell epitopes that are recognized by the antibody that dominantly cross-reacts with PAc.[30,40,41] On the basis of these findings, it is highly possible that the peptide is a multiple antigen that demonstrates strong immunogenicity and is effective for the induction of an anti-PAc antibody in humans.

The purpose of this study was to synthesize PAc peptide capable of binding with high affinity to most or all DR alleles, and to modify further such a PAc DR-binding peptide to create neutralizing antibodies against dental caries. The identification of the MHC binding motif is necessary to avoid negative residues that might not only lead to decreased affinity of a peptide for MHC but might also decrease the affinity for T-cell receptors (TCR). Therefore, we attempted to define the crucial DR contact residues as good binders, such as PAc(316–334) peptide. The binding motif common in DR8 (*DRB1*0802*), DR5 (*DRB1*1101*) and DR6 (*DRB1*1402* and **1405*) of the peptide was L—V—R— (Table 3). The amino acid is considered to be the common anchor that binds to these DR molecules. Frequently the motif of the peptide that combines with DR molecules is AxxxBxC. A variety of amino acids are located at the position A, B and C.[37,42] Specifically, A is an amino acid that plays an important role in high-affinity interactions between DR molecules and peptides. This amino acid demonstrates aromatic or large aliphatic peptide side chains, which interact with pockets in deep DR-binding grooves.[43,44] In the present study, it is considered that the amino acid equivalent to position A is L, position B is equivalent to V, and position C is equivalent to K. It is also suggested that the sequence L-RV-K–A, including relative position 3, R, and position 9, A, in addition to the common anchor is a multiple binding motif in HLA-DR8 (*DRB1*0802*), DR5 (*DRB1*1101*) and DR6 (*DRB1*1402* and **1405*) (Table 3).

It is very important to design a more effective peptide vaccine to prevent dental caries in humans, by combining B-cell epitopes that are recognized by neutralizing antibodies against dental caries using this kind of binding motif. In a

Table 3. Allele-specific motif of the DR-bound peptide

	Relative position								
	1	2	3	4	5	6	7	8	9
DRB1 0802*	**L**	**A**	**R**	**V**	**Q**	**K**	**A**	**N**	**A**
DRB1 1101*	**L**	**A**	**R**	**V**	**Q**	**K**	**A**	**N**	**A**
DRB1 1401*	**L**	**A**	**R**	**V**	**Q**	**K**	**A**	**N**	**A**
DRB1 1405*	**L**	**A**	**R**	**V**	**Q**	**K**	**A**	**N**	**A**

The specific binding sites demonstrated in this study are indicated in bold.

previous study, we have reported that the core of the B-cell epitope recognized by the antibody that inhibits the binding between PAc and salivary components is -Y——Y—- in PAc(365–377) peptide.[45] The core sequence of the amino acid is also included in PAc(301–319) peptide, which can induce antibodies that inhibit the colonization of *S. mutans* as described by Takahashi *et al.*[13] by immunization. It is suggested that such an amino acid sequence as -Y——Y— might play an important role in inhibiting the binding between PAc and salivary components and the colonization of *S. mutans*. Moreover, for inducing an inhibiting antibody with stable antigen peptides, a sequence such as -Y——Y—- may play an important role in maintaining the high-dimension structure.[45,46] However, PAc(301–319) and PAc(361–379) peptides bound strongly to the HLA-DR molecule in only two and three of nine subjects, respectively (Table 2). Therefore, these two peptides would not be effective as a dental caries vaccine against many MHC types seen in human. Recently, it has been reported that a hybrid peptide in which the central part of the epitope was completely replaced with pathogen-derived peptide fragments, including agretopes at both ends of the epitopes, could interact with the MHC molecule and induced an epitope-specific T-cell response.[47,48] This concept is called the 'cassette theory'. It has been reported that the hybrid peptide vaccine, prepared on the basis of this theory, could induce just the specific antibody for the sequence derived from the inserted pathogen.[49,50] It is also reported that high levels of neutralized antibody were induced by immunization of a coupled multiple peptide containing T-cell epitope in various MHC haplotypes and neutralizing epitopes.[19,21] These results suggest that a peptide vaccine with fewer adverse side-effects might be feasible. Therefore, it is considered that a peptide vaccine for dental caries that would be more effective in humans, with fewer adverse side-effects, could be designed by combining the sequence L-RV-K—A, clarified in the present experiment, with peptides that contain the core B-cell epitope (sequence -Y——Y—-) to produce only the inhibiting antibody against dental caries and maintain more stable peptide structures. Moreover, it is expected that such peptide design would provide useful information for designing peptide vaccines for infectious diseases in general.

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