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Identification of human T-cell epitopes and highly immunogenic analog peptides on the non-typeable *Haemophilus influenzae* P6 outer membrane protein

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Abstract P6 outer membrane protein is one of the candidates for a vaccine formulation against non-typeable *Haemophilus influenzae* (NTHi) infection. However, otitis-prone children who have recurrent episodes of acute otitis media due to NTHi fail to respond adequately to P6. An innovative approach to vaccination is therefore required to augment such children's immune response. To develop an effective peptide vaccine, we established P6-specific $CD4^+$ T-cell lines (TCLs) restricted by the human histocompatibility leukocyte antigen (HLA)-DR9 molecule, and revealed a human T-cell epitope on P6 and its core peptide sequence (p77–85; EYNIALGQR). Furthermore, we found that 3 analog peptides, E77D (the substitution of E at position 77 with D), N79G, and R85K, induced high proliferative responses as well as marked cytokine production when compared to the T-cell epitope peptide. These peptides may be candidates for a peptide vaccine formulation effective against NTHi infections, even in otitis-prone children.

Introduction

Non-typeable *Haemophilus influenzae* (NTHi) plays a major role in recurrent episodes of acute otitis media during childhood [1]. In addition, NTHi is the most common bacterial cause of exacerbations of chronic obstructive pulmonary disease (COPD) in adults [2]. In recent years,

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the prevalence of antibiotic-resistant NTHi has become an important issue. Significant interest therefore exists in developing a vaccine to prevent infections caused by NTHi in both children and adults.

Several outer membrane proteins of NTHi are reported to be targets for bactericidal antibody [1,3]. One of these proteins, P6, is a 16,000-Dalton lipoprotein that is highly conserved among strains and antigenically stable [4,5]. Antibody to P6 is detected in sera, middle ear effusions, nasopharyngeal secretions, and breast milk [6,7]. Anti-P6 antibody in nasopharyngeal secretions and breast milk is associated with a reduction in colonization with NTHi [8,9]. P6 is therefore proposed as a possible candidate for a vaccine formulation to prevent NTHi infections. In murine models, intranasal immunization with P6 and cholera toxin evokes a good P6-specific mucosal IgA antibody response, resulting in protection against NTHi [10–12]. However, otitis-prone children and some COPD patients fail to respond appropriately to P6 [6,13]. Although, as an immunogen, P6 activates peripheral blood lymphocytes in vitro in healthy adults [14], adenoidal lymphocytes from otitis-prone children show an impaired proliferative response to P6 [15]. In COPD patients, lower proliferative responses to P6 in peripheral T-cells are associated with frequent exacerbations due to NTHi [13]. These findings suggest that hyporesponsiveness to P6 in otitis-prone children and COPD patients might account for recurrent infections. The poor response to P6 is attributed to weak immunogenicity or selective immunologic derangements in such populations. Enhancement of the immunogenicity of P6 is therefore a key strategy in the development of a P6 vaccine.

Recently, treatment with peptides that contain T-cell and/or B-cell epitopes without excessive portions of antigenic protein was reported to induce effective immune responses without serious side effects [16]. In this system, T-cell epitope peptides directly combine with human histocompatibility leukocyte antigen (HLA)-class II on antigen presenting cells (APCs). $CD4^+$ T-cells then recognize these complexes and are activated. The activated $CD4^+$ T-cells help B-cells differentiate into plasma cells that produce specific antibody. Moreover, B-cell epitope peptides directly combine with HLA-class II on memory B-cells, causing B-cells to differentiate into plasma cells. Several experiments in murine models have identified epitopes responsible for the immune response to various infections [17–21]. However, as T- and B-cell epitopes of humans are not identical to those of mice [22], epitope analyses are necessary to develop peptide vaccines for clinical use.

The purpose of this study was to determine T-cell epitopes on P6 and to identify the highly immunogenic analog peptides of P6 in humans.

Materials and methods

Cell lines

Epstein–Barr virus (EBV)-transformed B lymphoblastoid cell lines (LCLs) were established from peripheral blood mononuclear cells (PBMCs) of HLA-typed volunteers by culturing with supernatant from the EBV-producing cell line B95-8 (American Type Culture Collection, Manassas, VA, USA). These cell lines were used as APCs.

Subjects

Seventeen healthy adult volunteers were enrolled in this study. HLA typing was carried out using a standard method at the Hokkaido Red Cross Blood Center (Sapporo, Japan). Informed consent for blood donation and HLA typing was obtained from all volunteers.

Purification of P6 and synthetic peptides

P6 was purified from NTHi strain 1479 as previously described, with some modifications [23–25]. In the purified P6 preparation, endotoxin (lipopolysaccharide; LPS) was not detected by TOXICOLOR® (Seikagaku Corporation, Tokyo, Japan) and lipooligosaccharide (LOS) was not found on limulus amebocyte assay using the E-Toxate kit (Sigma, St. Louis, MO, USA). To analyze T-cell epitopes, 13 sequential 15-mer peptides, which overlapped by five residues and

Table 1 Sequence of 13 overlapping peptides and control peptide

Region of P6 protein	Amino acid sequence (one letter code)														
p1–15	C	S	S	S	N	N	D	A	A	G	N	G	A	A	Q
p11–25	N	G	A	A	Q	T	F	G	G	Y	S	V	A	D	L
p21–35	S	V	A	D	L	Q	Q	R	Y	N	T	V	Y	F	G
p31–45	T	V	Y	F	G	F	D	K	Y	D	I	T	G	E	Y
p41–55	I	T	G	E	Y	V	Q	I	L	D	A	H	A	A	Y
p51–65	A	H	A	A	Y	L	N	A	T	P	A	A	K	V	L
p61–75	A	A	K	V	L	V	E	G	N	T	D	E	R	G	T
p71–85	D	E	R	G	T	P	E	Y	N	I	A	L	G	Q	R
p81–95	A	L	G	Q	R	R	A	D	A	V	K	G	Y	L	A
p91–105	K	G	Y	L	A	G	K	G	V	D	A	G	K	L	G
p101–115	A	G	K	L	G	T	V	S	Y	G	E	E	K	P	A
p111–125	E	E	K	P	A	V	L	G	H	D	E	A	A	Y	S
p121–134	E	A	A	Y	S	K	N	R	R	A	V	L	A	Y	
YF (control peptide)	G	A	M	R	V	T	K	D	T	N	D	N	N	L	Y

covered the entire sequence of P6 [26], were prepared (Table 1). A 15-mer control peptide was also synthesized based on the arbitrary sequence of yellow fever [27]. These peptides were synthesized using the Fmoc solid-phase method (SAWADY Technology, Tokyo, Japan).

In vitro generation of dendritic cells (DCs)

DCs were used as APCs, and these were generated from CD14-positive monocytes as previously reported [28]. PBMCs from healthy donors were isolated by the gradient centrifugation method (Ficoll Paque Plus®; Amersham Pharmacia Biotech, Piscataway, NJ, USA). CD14-positive monocytes were purified from PBMCs by positive immunoselection using an anti-CD14 antibody coupled onto magnetic microbeads (MACS®; Miltenyi Biotec, Auburn, CA, USA). The CD14-positive monocytes were cultured at 1×10^6 cells/ml in the presence of 50 ng/ml of IL-4 (PeproTec, London, UK) and 50 ng/ml of GM-CSF (PeproTec, London, UK) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Equitech-bio, Ingram, TX, USA), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin G (PCG), and 100 μg/ml streptomycin. After 7 days' culture, the nonadherent cells were harvested and used as APCs.

In vitro lymphocyte proliferation assay

An in vitro lymphocyte proliferation assay was performed as reported previously [28]. DCs were cultured with 10 μg/ml P6 protein or 5 μg/ml synthetic overlapping peptide for 2 h at 37°C in a humidified 5% CO_2 atmosphere. Concentrations of antigens were determined based on the results of our previous study [28]. After irradiation (4000 rads), 5×10^3 DCs were cultured with 1×10^5 autologous $CD4^+$ T-cells, which were purified by positive immune selection with anti-CD4 antibody coupled onto magnetic microbeads. This was performed in 200 μl of RPMI1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2×10^{-5} 2ME, and 10% heat-inactivated human AB serum (SIGMA-ALDORICH, St. Louis, MO, USA) for 7 days at 37°C, in a humidified 5% CO_2 atmosphere. As a mitogen control, 5 μg/ml phytohemagglutinin (PHA; SIGMA-ALDORICH, St. Louis, MO, USA) was then added to the corresponding wells 72 h before harvest. Eighteen hours before harvest, 0.5 μCi/well [^{3}H]-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, England) was added to each well. Cells were then harvested on glass-fiber filters and the incorporated radioactivity of harvested cells was measured by liquid scintillation counting. The proliferative response was expressed as a stimulation index (SI) and the cutoff for a positive response was set at 2.5, as explained in our previous study [29]. The SI was calculated by dividing the mean radioactivity (cpm) obtained in the presence of antigen by the mean radioactivity (cpm) obtained in the absence of antigen but in the presence of APCs.

Antigen-specific T-cell lines (TCLs)

P6-specific TCLs were established from 2 healthy donors (no. 4 and 8: DR8/9 positive) as reported previously, with some modifications [28]. P6 (1 μg/ml)-pulsed DCs (5×10^3/well) were irradiated (4000 rads) and cultured with 5×10^4 /well autologous $CD4^+$ T-cells in 200 μl of AIM-V (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 3% heat-inactivated human male AB serum for 7 days at 37°C, in a humidified 5% CO_2 atmosphere. Secondary cultures were set up by the addition of irradiated (4000 rads) autologous PBMCs (1×10^5/well) pulsed with P6 (1 μg/ml) to the primary 7-day culture. Two days after the second stimulation, human recombinant IL-2 (SHIONOGI and CO., LTD., Osaka, Japan) was added to each well at a final concentration of 10 IU/ml. One week later, the specificity of TCLs was determined using [^{3}H]-thymidine incorporation with irradiated (4000 rads) PBMC pulsed with P6. TCLs were maintained by weekly stimulation with irradiated autologous PBMCs (1×10^6 /well) pulsed with P6 (1 μg/ml) in the same medium containing 25 IU/ml IL-2.

T-cell epitope and HLA restriction

The T-cell epitope and the core peptide sequence of TCLs were confirmed as described previously [29]. TCLs (5×10^4 /well) were cultured with irradiated (4000 rads) autologous PBMCs (1×10^5/well) pulsed with overlapping or truncated peptides (5 μg/ml) in triplicate in 96-well culture plates for 72 h at 37°C, in a humidified 5% CO_2 atmosphere, after which the proliferative responses were measured.

To determine the HLA restriction involved in antigen presentation, the following 2 experiments were performed as described previously [29]. (i) TCLs (3×10^4/well) were cultured with irradiated (4000 rads) autologous PBMCs (1×10^5/well) and pulsed with peptide (5 μg/ml) in the presence or absence of various mouse monoclonal antibodies (mAbs) specific to HLA molecules, anti-HLA-DR (BD Biosciences, San Diego, CA, USA), anti-HLA-DQ (IMMUNOTECH, Marseille, France), and anti-HLA-class I (IMMUNOTECH, Marseille, France) for 72 h at 37°C, in a humidified 5% CO_2 atmosphere, after which the proliferative responses were measured. All antibodies were used at final concentrations of 10 μg/ml. (ii) TCLs (3×10^4 /well) were cultured with irradiated (8000 rads) LCLs (2×10^4/well), which were established from different HLA-typed volunteers, pulsed with peptide (5 μg/ml) for 72 h at 37°C, in a humidified 5% CO_2 atmosphere, after which the proliferative responses were measured. The numbers of cells in each experiment were determined based on the results of a previous study (data not shown) [29].

Cytokine analysis of TCLs

For the measurement of cytokines, TCLs (5×10^5 /well) and irradiated autologous PBMCs (1×10^6/well) pulsed with P6 (1 μg/ml) or various peptides (5 μg/ml) were co-cultured in 24-well plates. Culture supernatants were harvested after 0, 12, 24, 48, and 72 h and stored at −70°C before the assays. Cytokine levels in culture supernatants were measured using a solid-phase enzyme-linked immunosorbent assay (ELISA) with BD OptEIA™ Set Human interferon-gamma (IFN-γ), interleukin (IL)-4, IL-5, IL-6, and transforming growth factor (TGF)-β (BD Biosciences, San Diego, CA, USA).

Results and discussion

T-cell responses to overlapping synthetic peptides

To identify the human T-cell epitope of P6, we first investigated the T-cell responses to 13 different overlapping peptides in 17 healthy donors. As shown in Fig. 1, all healthy donors showed strong responses to P6 and PHA as mitogen controls, but none responded to yellow fever (YF) peptide as a control peptide. Six donors (no. 2, 4, 8, 12, 15, and 17) expressed HLA-DR4 and/or HLA-DR9 and responded to several overlapping peptides (p41—55, p51—65, and p71—85) known to contain HLA-DR4 or HLA-DR9 binding motifs. [30—32]. In 2 donors who expressed HLA-DR8/9, peptides induced proliferative responses with SI $\geq$ 2.5 for 5 peptides in donor no. 8 and 7 peptides in donor no. 4 (including four peptides with SI $\geq$ 5). Hence, both donors expressing HLA-DR9 responded to p41—55, p51—65, and p71—85, indicating that these peptides contained a T-cell epitope of P6 restricted to HLA-DR9-positive individuals.

Proliferative responses of TCLs to overlapping peptides

Of the 13 overlapping peptides covering the entire sequence of P6, in vitro proliferative responses of YAS-TCL and YILY-TCL established from donor nos. 4 and 8, respectively, were unique (Fig. 2). Only p51—65 in YAS-TCL and p71—85 in YILY-TCL produced proliferative responses with SI greater than 5, showing that the T-cell epitopes of these two TCLs are localized. YAS-TCL responded to both P6 and p51—65 in a dose-dependent manner (Fig. 2C). Likewise, YILY-TCL responded to both P6 and p71—85 in a dose-dependent manner (Fig. 2D). Both proliferative responses were inhibited by anti-HLA-DR antibody and found in co-cultures with LCLs that expressed HLA-DR9 (Fig. 3). The sequences of these 2 peptides contained a HLA-DR9-binding motif [30,31], suggesting that T-cell epitopes of P6 restricted by HLA-DR9 are present in p51—65 and p71—85. In murine models, several mouse B-cell epitopes of P6 have already been identified [22]. To our knowledge, however, this is the first report of a human T-cell epitope on P6. Aside from the TCL donors, 2 (nos. 12, 15) and 4 (nos. 12, 15, 2, 17) of 8 DR4-positive individuals recognized p51—65 and p71—85, respectively (Fig. 1). On the other hand, 3 of 6 DR9-positive individuals did not respond to these two DR9 restricted T-cell epitopes. These two T-cell epitopes may be cryptic for these donors. Otherwise, they may respond to other DR9 restricted epitopes or their response may be restricted to other HLA class II or other DR phenotypes. Although all the HLA binding motifs have not been clarified, P6 protein contains 9 DR4-binding motif, 5 DR4-binding motif and 4 DR15-binding motif [30,31]. On the other hand, all of the DR15-positive individuals responded to either p51—65 or p71—85 (Fig. 1), and an immune response was induced in 12 of 17 donors (70.5%), making p51—65 and p71—85 candidates for a P6 peptide vaccine. Total prevalence of HLA-DR4, 9, and 15 is over 50% in the Japanese population [33]. Recently, we have established that a human B-cell epitope is also located on p71—85 (data not shown); therefore, p71—85 is a more likely candidate for a vaccine formulation because it contains T- and B-cell epitopes. We focused on this segment in subsequent analyses of the core peptide sequence and the highly immunogenic analog peptide.

HLA class II restriction of TCLs

HLA class II restriction of YILY-TCL and YAS-TCL was determined by analyzing the proliferative response to p71—85 or p51—85 culturing with irradiated autologous PBMCs and anti-HLA antibodies (Fig. 3). Monoclonal Abs to either HLA-class I or HLA-DQ did not affect the specific

	No. of donors (HLA-DR phenotype)																
	9 (15/16)	7 (4/8)	11 (12)	10 (8/15)	8 (8/9)	12 (4/9)	4 (8/9)	1 (15/13)	5 (15/14)	15 (4)	3 (15)	2 (4/12)	17 (4/13)	14 (15)	13 (4/9)	16 (9/14)	6 (4/9)
P6	127.4	125.8	431.4	270.5	10.3	32.1	594.5	469.3	45.9	190.9	28.5	63.7	88.1	40.6	70.4	476.0	42.8
p1-15																	
p11-25																	
p21-35																	
p31-45																	
p41-55																	
p51-65																	
p61-75																	
p71-85																	
p81-95																	
p91-105																	
p101-115																	
p111-125																	
p121-134																	
YF																	
PHA	514.1	248.2	705.2	826.2	11.8	23.7	603.0	176.6	70.7	180.0	104.0	96.4	141.9	106.2	130.9	678.6	708.3
Antigen Free	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Figure 1 Proliferative responses to 13 overlapping peptides and P6 in $CD4^+$ T-cells from 17 healthy donors. The first column indicates the amino acid position of the synthetic peptide. The donor numbers and HLA phenotypes are shown in the first row. Proliferative responses are expressed as SI (defined as cpm in the presence of antigen/cpm in the absence of antigen) and are shown in the boxes. White boxes indicate SI<2.5, gray boxes indicate 2.5≤SI<5.0, and black boxes indicate SI≥5.0, (higher than that of control peptide (YF)). YF: yellow fever, PHA: phytohemagglutinin.

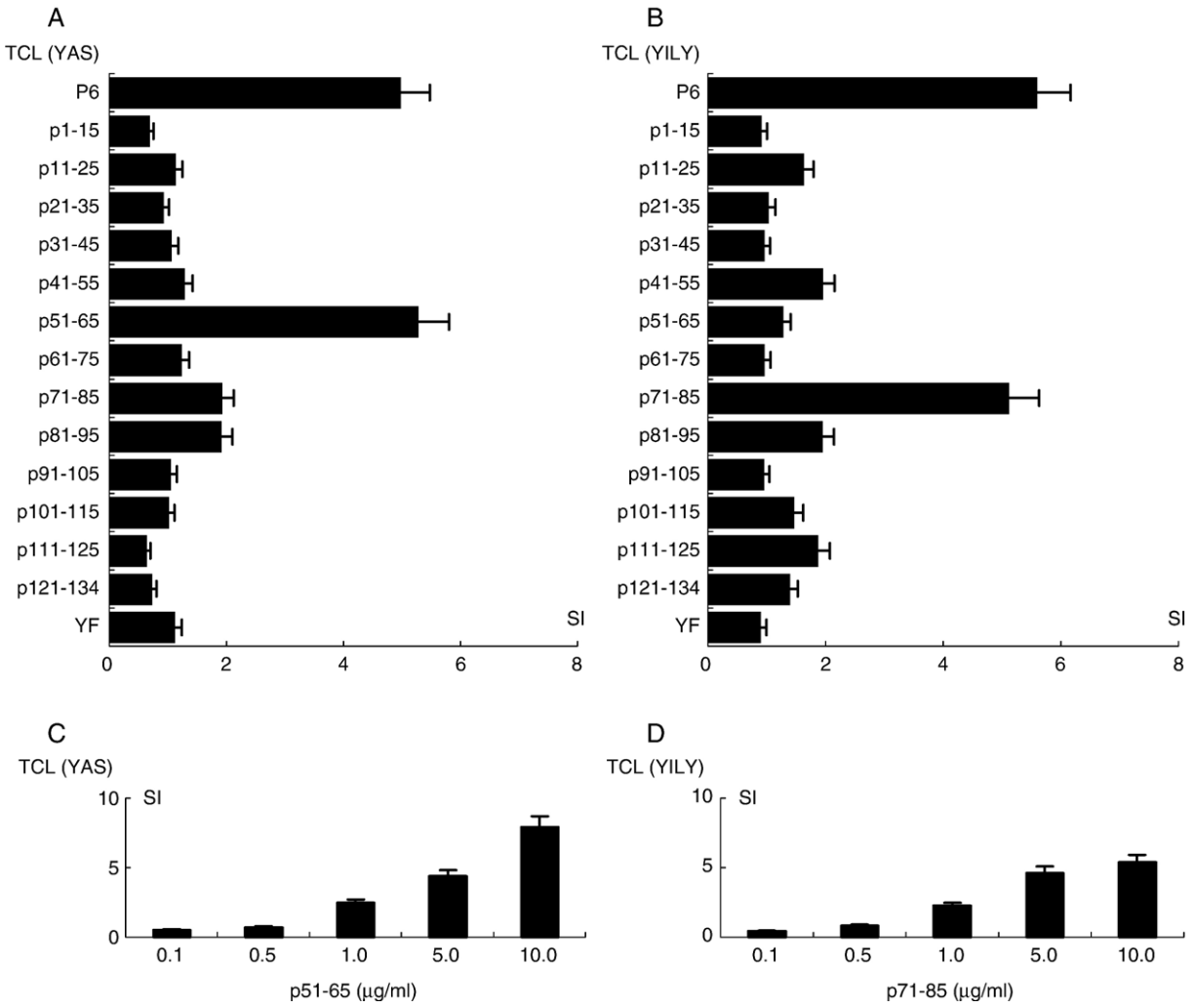

Figure 2 Proliferative responses to 13 overlapping peptides in two P6-specific T-cell lines (TCLs) (A and C, YAS; and B and D, YILY) established from HLA-DR8/9-positive individuals (donors no. 4 and 8, respectively; Table 1). The first column indicates the amino acid position of the synthetic peptide. Bars show proliferative responses expressed as SI (defined as cpm in the presence of antigen/cpm in the absence of antigen) and error bars represent standard error. In YILY-TCL established from donor no. 8, only the p71—85 peptide induced proliferative responses with SI greater than 5 (B). YAS-TCL responded to both P6 and p51—65 in a dose-dependent manner (C). Likewise, the YILY-TCL peptide responded to both P6 and p71—85 in a dose-dependent manner (D). YF: yellow fever.

proliferative response, whereas anti-HLA-DR inhibited the response (Figs. 3A and C). The proliferative response of peptide was then analyzed in cultures with 7 irradiated LCLs expressing different HLA-DR types. Strong proliferative responses to peptides were found in autologous LCL (DR8/9) and DR4/9 and DR9/9, which all expressed HLA-DR9 (Figs. 3B and D). These findings suggest that the proliferative responses of YILY-TCL and YAS-TCL were restricted to the HLA-DR9 molecule.

Core peptide sequence recognition by TCL

The core peptide sequence of P6 recognized by YILY-TCL [29] was analyzed using 14 truncated peptides synthesized based on p71—85 with conservative and non-conservative amino acid substitutions (Fig. 4). The proliferative response of YILY-TCL decreased when the peptide was truncated either from the N terminus to glutamine at position 77 or from the C terminus to arginine at position 85. These results indicate that the core peptide sequence recognized by YILY-TCL is the amino acid sequence p77—85 (EYNIALGQR). It has been previously observed that T-cells do not proliferate maximally with an 8- or 9-mer core peptide, and several additional residues at both the N- and the C-terminus are required for a full response [34]. Because in general CD4+T-cells prefer to recognize peptides of at least 15 residues [28], the 15-mer amino acid sequence p74—88 (GTPEYNI ALGQRRAD) was used for subsequent study.

Proliferative responses of TCL to substituted peptides

To determine the amino acid residue on p74—88 (GTPEYNIALGQRRAD) responsible for T-cell activation and HLA binding, we analyzed proliferative responses of YILY-TCL to 9 analog peptides whose sequences contained an amino acid

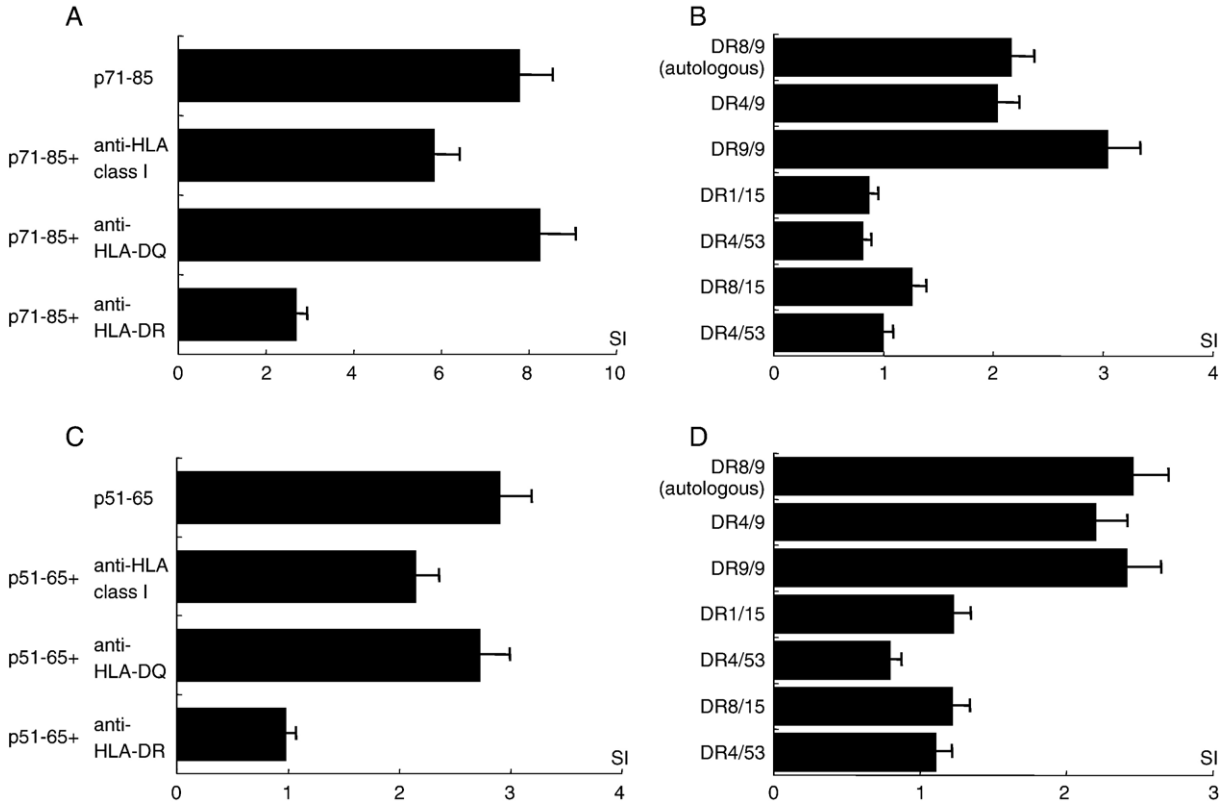

Figure 3 P6-specific proliferative responses of the T-cell line (TCL) YILY cultured with irradiated autologous peripheral mononuclear cells in the presence or absence of antibodies to HLA molecules (A) and with irradiated lymphoblastoid cell lines (LCLs) established from different HLA-typed volunteers (B). Bars show proliferative responses expressed as SI (defined as cpm in the presence of antigen/cpm in the absence of antigen) and error bars represent standard errors. Antibodies to HLA-class I or HLA-DQ did not affect the P6-specific proliferative response, whereas anti-HLA-DR inhibited it (A). Proliferative response to P6 was found in cultures with autologous LCL (DR8/9) and 2 LCLs (DR4/9 and DR9/9), but was not found in cultures with LCLs established from non-HLA-DR9 holders (B). These results suggest that the proliferative response of YILY-TCL was restricted by HLA-DR9 molecules. Similar results were obtained for YAS-TCL (C, D).

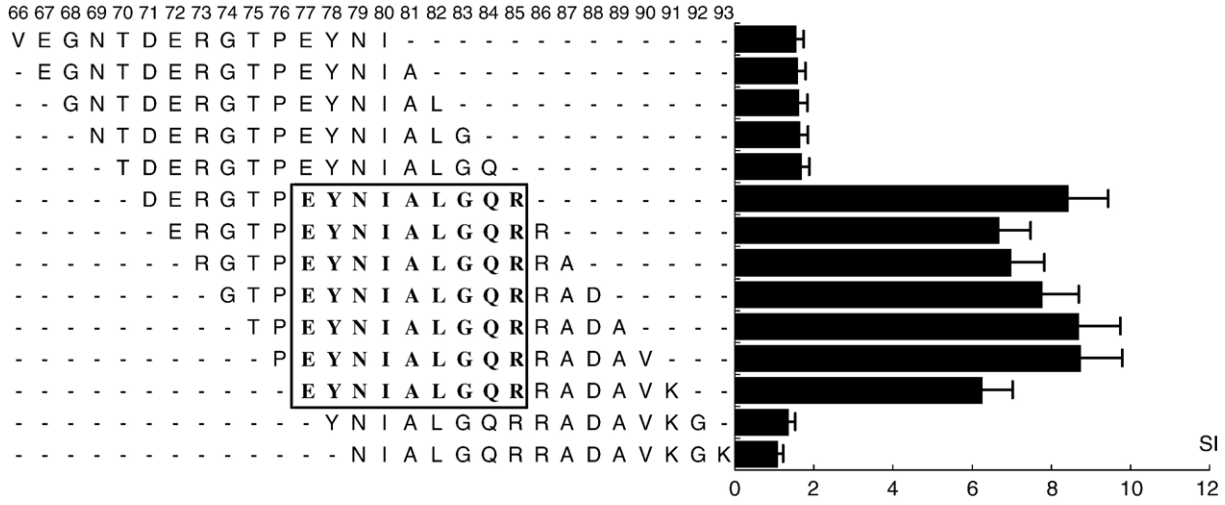

Figure 4 Analysis of the core peptide sequence recognized by the P6-specific T-cell line (TCL) YILY. Proliferative responses of YILY-TCL to 14 truncated peptides, which were offset by one amino acid based on p71—85, were analyzed. Bars show proliferative responses expressed as SI (defined as cpm in the presence of antigen/cpm in the absence of antigen) and error bars represent standard errors. A single-letter code for amino acids is used. The proliferative response found on stimulation with the peptides containing the common sequence p77—85 is shown in bold letters. This sequence (EYNIALGQR) corresponds to the core peptide sequence recognized by YILY-TCL.

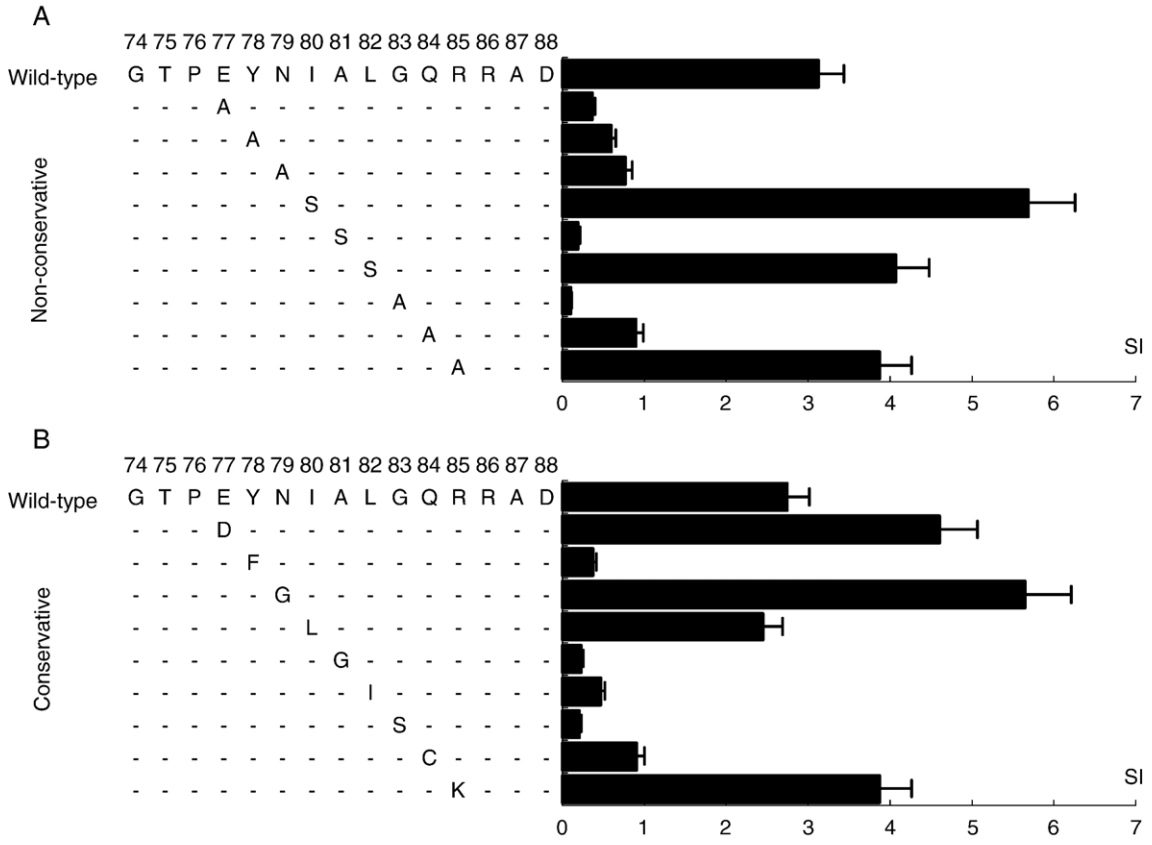

Figure 5 Proliferative response of the P6-specific T-cell line (TCL) YILY to analog peptides with non-conservative (A) and conservative (B) amino acid substitutions in the T-cell epitope sequence p77—85 (EYNIALGQR). Bars show proliferative responses expressed as SI (defined as cpm in the presence of antigen/cpm in the absence of antigen) and error bars represent standard errors. The upper amino acid sequence shows the wild-type peptide sequence. Single letters indicate the site of the substitution and dashes indicate amino acids identical to the wild-type peptide.

substitution in the core peptide sequence p77—85 (EYNIALGQR). These non-conservative amino acid substitutions were carried out as previously described [29]. Hydrophilic residues (tyrosine, glycine, arginine, aspartic acid, glutamine, and glutamic acid) were substituted with the small hydrophobic residue alanine. Hydrophobic residues (alanine, leucine, and isoleucine) were substituted with the small hydrophilic residue serine. As shown in Fig. 5A, proliferative responses to the peptide E77A (indicates the substitution of E: glutamine, at position 77, with A: alanine) (SI = 0.36); to Y78A (SI = 0.59); to N79A (SI = 0.77); to A81S (SI = 0.19); to G83A (SI = 0.10); and to Q84A (SI = 0.89) were

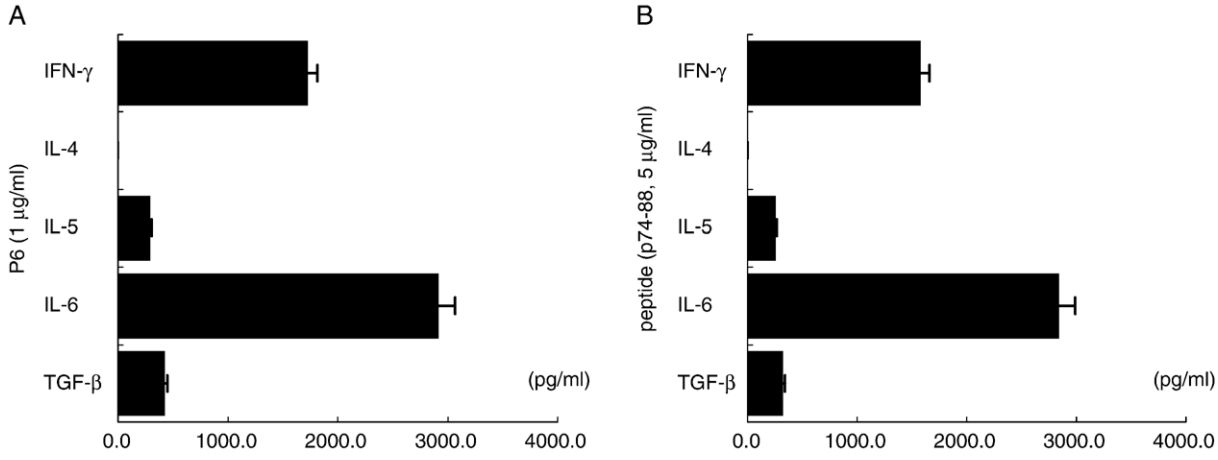

Figure 6 Cytokine production in the P6-specific T-cell line (TCL) YILY stimulated with P6 (A) and the peptide p74—88 (B). Cytokine levels in culture supernatants at 48 h after stimulation with P6 (A) and wild-type peptide; p74—88 (B) levels were assayed using ELISA. Bars show cytokine concentrations and error bars represent standard errors.

significantly lower than that to the wild-type peptide (SI = 3.1). On the other hand, the proliferative response to I80S (SI = 5.6) was greater than that to the wild-type peptide (SI = 3.1). Since 78Y (tyrosine) and 81A (alanine) were reported as the HLA-DR9-binding motif [31], these two residues are considered important for HLA class II binding. On the other hand, the remaining 4 residues; 77E (glutamine), 79N (aspartic acid), 83G (glycine), and 84Q (glutamic acid), are likely to be important for recognition by the T-cell receptor and for T-cell activation. Interestingly, the proliferative response to I80S was greater than that to the wild-type peptide (Fig. 5A). This substitution might change the structure and exert a positive influence on the TCR site without any alteration of HLA-binding ability.

To obtain a more immunogenic analog peptide, we also carried out chemically conservative amino acid substitution on the core peptide sequence p77—85 (EYNIALGQR), as described previously [29]. Alanine, aspartic acid, and glutamic acid were substituted with glycine, and leucine and isoleucine were substituted for each other. Likewise, phenylalanine and tyrosine, lysine and arginine, glutamine and asparagine, and glycine and serine were substituted for each other. As shown in Fig. 5B, the proliferative response of YILY-TCL to 3 substituted peptides, E77D (SI = 4.6), N79G (SI = 5.6), and R85K (SI = 3.8), was greater than that to wild-type peptide (SI = 2.5). On the other hand, I80L (SI = 2.4) did not affect the response and Y78F (SI = 0.38), A81G (SI = 0.23), L82I (SI = 0.47), G83S (SI = 0.20), and Q84C (SI = 0.97) diminished it.

Cytokine production of TCL in response to P6 and the epitope p74–88

To determine the cytokine profile produced by YILY-TCL, we measured IFN-γ, IL-4, IL-5, IL-6, and TGF-β at 0, 12, 24, 48, and 72 h after stimulation with P6 or the peptide p74—88 (GTPEYNIALGQRRAD). Doses of antigen were optimized with proliferative responses of YILY-TCL because cpm of YILY-TCL were almost equal to those of P6 (1 μg/ml) and peptides (5 μg/ml) (Fig. 2B). These cytokines were not detected at time 0 or in the absence of antigens (data not shown). As shown in Fig. 6, P6 and p74—88 induced IFN-γ, IL-5, IL-6, and TGF-β production. Cytokine levels after p74—88 stimulation were equal to those after P6 treatment; IFN-γ and IL-6 concentrations were several thousand pg/ml and IL-5 and TGF-β concentrations were several hundred pg/ml. IL-4 was not detected at any culture duration for stimulation with P6 or p74—88. Since the cytokine levels and profile of YILY-TCL were almost the same with P6 and p74—88 stimulations, the epitope peptide is equivalent to P6 in its antigenicity.

Interestingly, the cytokine profile of YILY-TCL corresponds to that found in a murine study by Kodama et al. [11], in which numerous P6-specific IgA-producing cells and CD4 T-cells expressing mRNA of IFN-γ, IL-5, IL-6, and TGF-β were found in the middle ear mucosa of mice immunized intranasally with P6 and cholera toxin [11]. A number of studies also showed that IFN-γ, IL-5, IL-6, and TGF-β play an important role in IgA antibody production [35—38]. Hiroi et al. [35] demonstrated increases in the number of mouse IgA-producing cells isolated from the submandibular gland when IFN-γ-producing αβ T-cells and B-cells were co-cultured. Beagley et al. [36] showed that IL-5 induced Peyer's patch IgA^+ B-cell blasts to differentiate into IgA synthesis. They also showed that IL-6 promoted the terminal differentiation of Peyer's patch B-cells into IgA-secreting plasma cells [37]. Defrance et al. [38] showed that TGF-β induced human tonsillar B-cells to synthesize IgA. Secretory IgA antibody is known to play a very important role in mucosal immunity because of its inhibitory effects on bacterial adherence. Intranasal immunization is now considered an effective route of vaccination for the induction of an anamnestic IgA response to NTHi or P6 [10—12,39]. On the other hand, IL-4 is reported to induce production of IgE [40] and anaphylaxis, but IL-4 secretion was not detected in our experiments. On the basis of these findings, it is suggested that the cytokine profile induced by p74—88; i.e., IFN-γ, IL-5, IL-6, and TGF-β without IL-4, is likely to be advantageous for the induction of a P6-specific IgA antibody response without anaphylaxis.

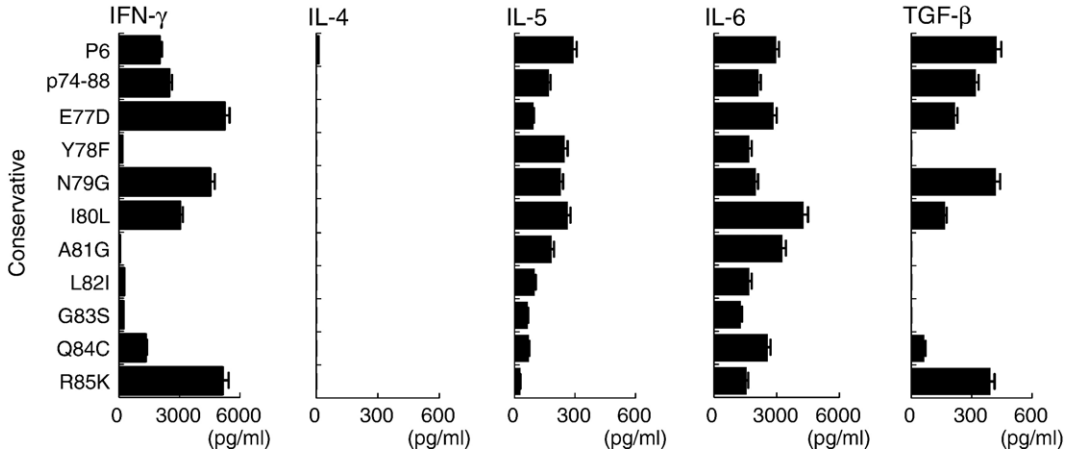

Figure 7 Cytokine production in the P6-specific T-cell line (TCL) YILY stimulated with analog peptides with conservative amino acid substitutions in the T-cell epitope sequence p77—85 (EYNIALGQR). The letters in the first column consecutively indicate the original amino acid, its position, and the substituted amino acids of the analog peptide (e.g. E77A indicates the substitution of E at position 77 with A). Bars show cytokine concentrations and error bars indicate standard errors.

Cytokine production of TCL in response to the substituted peptides

To analyze more effective peptide antigens, we measured cytokine production of YILY-TCL in response to chemically conservative substituted peptides (Fig. 7). Analog peptides induced various changes of YILY-TCL cytokine profile. Although several analog peptides induced higher IFN-γ, IL-5, IL-6, and TGF-β production than wild-type peptide p74—88, no analog peptide induced IL-4 production. The analog peptides E77D (5223 pg/ml), N79G (4514 pg/ml), and R85K (5164 pg/ml) enhanced IFN-γ production compared to that of the wild-type peptide p74—88 (2504 pg/ml). The production of IL-5 was greater on stimulation with the analog peptides Y78F (249 pg/ml), N79G (227 pg/ml), and I80L (263 pg/ml) than with wild type peptide (171 pg/ml). The production of IL-6 was greater on stimulation with the analog peptides E77D (2844 pg/ml), I80L (4274 pg/ml), and A81G (3273 pg/ml) than with wild type peptide (2130 pg/ml). The production of TGF-β was greater on stimulation with the analog peptides N79G (419 pg/ml) and R85K (394 pg/ml) than with wild-type peptide (318 pg/ml).

The stronger proliferative response to the analog peptides E77D, N79G, and R85K than to the wild-type peptides suggest that these substituted peptides may be more advantageous for T-cell recognition and activation. They might also promote greater cytokine production than the T-cell epitope peptide with antigenicity equivalent to P6, evoking a stronger P6-specific IgA antibody response.

Conclusions

We identified one of the human T-cell epitopes of P6, p77—85 (EYNIALGQR); it is restricted by HLA-DR9, which is frequently found in Japanese individuals [33]. In cytokine analyses, P6 and the T-cell epitope peptide were found to induce the production of cytokines such as IFN-γ, IL-5, IL-6, and TGF-β, but not IL-4. This cytokine profile is considered to be advantageous for inducing anamnestic IgA antibody responses [35—38]. Furthermore, 3 analog peptides, E77D, N79G, and R85K, were found to induce greater proliferative responses and cytokine release than the T-cell epitope peptide with antigenicity equivalent to P6. The T-cell epitope peptide containing the sequence p77—85 and/or its immunogenic analog peptides E77D, N79G, and R85K might therefore be a candidate for a P6-peptide vaccine against NTHi infection, particularly in HLA-DR9-positive individuals. However, when compared to P6, T-cell responses to peptides are modest due to low antigenicity as single peptide vaccines. Therefore it is necessary to combine these analog peptides. Polypeptide vaccine [17,41,42] conjugated to these peptides may be useful in inducing immune responses and overcoming poor responses to P6 in otitis-prone children and COPD patients. To develop a vaccine formulation effective for a broad population, the present information that was mostly gathered from two cell lines is preliminary, further analyses of the T-cell epitopes recognized by multiple HLA class II molecules and in-vivo studies will be needed.

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References

[1] T.F. Murphy, M.A. Apicella, Nontypable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection, Rev. Infect. Dis. 9 (1987) 1–15.
[2] S. Sethi, T.F. Murphy, Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review, Clin. Microbiol. Rev. 14 (2001) 336–363.
[3] T.F. Murphy, L.C. Bartos, P.A. Rice, M.B. Nelson, K.C. Dudas, M.A. Apicella, Identification of a 16,600-dalton outer membrane protein on nontypeable *Haemophilus influenzae* as a target for human serum bactericidal antibody, J. Clin. Invest. 78 (1986) 1020–1027.
[4] T.F. Murphy, M.B. Nelson, K.C. Dudas, J.M. Mylotte, M.A. Apicella, Identification of a specific epitope of *Haemophilus influenzae* on a 16,600-dalton outer membrane protein, J. Infect. Dis. 152 (1985) 1300–1307.
[5] T.F. Murphy, L.C. Bartos, A.A. Campagnari, M.B. Nelson, M.A. Apicella, Antigenic characterization of the P6 protein of nontypable *Haemophilus influenzae*, Infect. Immun. 54 (1986) 774–779.
[6] N. Yamanaka, H. Faden, Antibody response to outer membrane protein of nontypeable *Haemophilus influenzae* in otitis-prone children, J. Pediatr. 122 (1993) 212–218.
[7] N. Yamanaka, H. Faden, Local antibody response to P6 of nontypable *Haemophilus influenzae* in otitis-prone and normal children, Acta Oto-laryngol. 113 (1993) 524–529.
[8] Y. Harabuchi, H. Faden, N. Yamanaka, L. Duffy, J. Wolf, D. Krystofik, Nasopharyngeal colonization with nontypeable *Haemophilus influenzae* and recurrent otitis media. Tonawanda/Williamsville Pediatrics, J. Infect. Dis. 170 (1994) 862–866.
[9] Y. Harabuchi, H. Faden, N. Yamanaka, L. Duffy, J. Wolf, D. Krystofik, Human milk secretory IgA antibody to nontypeable *Haemophilus influenzae*: possible protective effects against nasopharyngeal colonization, J. Pediatr. 124 (1994) 193–198.
[10] M. Hotomi, T. Saito, N. Yamanaka, Specific mucosal immunity and enhanced nasopharyngeal clearance of nontypeable *Haemophilus influenzae* after intranasal immunization with outer membrane protein P6 and cholera toxin, Vaccine 16 (1998) 1950–1956.
[11] S. Kodama, S. Suenaga, T. Hirano, M. Suzuki, G. Mogi, Induction of specific immunoglobulin A and Th2 immune responses to P6 outer membrane protein of nontypeable *Haemophilus influenzae* in middle ear mucosa by intranasal immunization, Infect. Immun. 68 (2000) 2294–2300.
[12] M. Hotomi, N. Yamanaka, J. Shimada, M. Suzumoto, Y. Ikeda, A. Sakai, J. Arai, B. Green, Intranasal immunization with recombinant outer membrane protein P6 induces specific immune responses against nontypeable *Haemophilus influenzae*, Int. J. Pediatr. Otorhinolaryngol. 65 (2002) 109–116.

[13] Y. Abe, T.F. Murphy, S. Sethi, H.S. Faden, J. Dmochowski, Y. Harabuchi, Y.M. Thanavala, Lymphocyte proliferative response to P6 of *Haemophilus influenzae* is associated with relative protection from exacerbations of chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 165 (2002) 967–971.
[14] H. Kodama, H. Faden, Cellular immunity to the P6 outer membrane protein of nontypeable *Haemophilus influenzae*, Infect. Immun. 63 (1995) 2467–2472.
[15] H. Kodama, H. Faden, Y. Harabuchi, A. Kataura, J.M. Bernstein, L. Brodsky, Cellular immune response of adenoidal and tonsillar lymphocytes to the P6 outer membrane protein of non-typeable *Haemophilus influenzae* and its relation to otitis media, Acta Otolaryngol. 119 (1999) 377–383.
[16] G. Ada, Vaccines and vaccination, N Engl. J. Med. 345 (2001) 1042–1053.
[17] P. Migliorini, B. Betschart, G. Corradin, Malaria vaccine: immunization of mice with a synthetic T cell helper epitope alone leads to protective immunity, Eur. J. Immunol. 23 (1993) 582–585.
[18] C. Olive, T. Clair, P. Yarwood, M.F. Good, Protection of mice from group A streptococcal infection by intranasal immunisation with a peptide vaccine that contains a conserved M protein B cell epitope and lacks a T cell autoepitope, Vaccine 20 (2002) 2816–2825.
[19] S.J. Barenkam, J.W. St Geme III, Identification of surface-exposed B-cell epitopes on high molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae*, Infect. Immun. 64 (1996) 3032–3037.
[20] L.A. Novotny, J.A. Jurcisek, M.E. Pichichero, L.O. Bakaletz, Epitope mapping of the outer membrane protein P5-homologous fimbrin adhesin of nontypeable *Haemophilus influenzae*, Infect. Immun. 68 (2000) 2119–2128.
[21] W.A. Hayman, I. Toth, N. Flinn, M. Scanlon, M.F. Good, Enhancing the immunogenicity and modulating the fine epitope recognition of antisera to a helical group A streptococcal peptide vaccine candidate from the M protein using lipid-core peptide technology, Immunol. Cell Biol. 80 (2002) 178–187.
[22] A.G. Beck-Sickinger, H. Rotering, K.H. Wiesmuller, F. Dorner, G. Jung, Mapping of antigenic and immunogenic sites of *Haemophilus influenzae* outer membrane protein P6 using synthetic lipopeptides, Biol. Chem. Hoppe-Seyler. 375 (1994) 173–182.
[23] R.S. Munson Jr., D.M. Granoff, Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae type b, Infect. Immun. 49 (1985) 544–549.*
[24] W.H. Badr, D. Loghmanee, R.J. Karalus, T.F. Murphy, Y. Thanavala, Immunization of mice with P6 of nontypeable *Haemophilus influenzae*: kinetics of the antibody response and IgG subclasses, Vaccine 18 (1999) 29–37.
[25] R.J. Karalus, T.F. Murphy, Purification and characterization of outer membrane protein P6, a vaccine antigen of non-typeable *Haemophilus influenzae*, FEMS Immunol. Med. Microbiol. 26 (1999) 159–166.
[26] M.B. Nelson, M.A. Apicella, T.F. Murphy, H. Vankeulen, L.D. Spotila, D. Rekosh, Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6, Infect. Immun. 56 (1988) 128–134.
[27] M.R. Pisano, J. Nicoli, H. Tolou, Homogeneity of yellow fever virus strains isolated during an epidemic and a post-epidemic period in West Africa, Virus Genes 14 (1997) 225–234.
[28] H. Kobayashi, M. Wood, Y. Song, E. Appella, E. Celis, Defining promiscuous MHC class II helper T-cell epitopes for the HER2/neu tumor antigen, Cancer Res. 60 (2000) 5228–5236.
[29] Y. Abe, S. Kimura, T. Kokubo, K. Mizumoto, M. Uehara, M. Katagiri, Epitope analysis of birch pollen allergen in Japanese subjects, J. Clin. Immunol. 17 (1997) 485–493.
[30] H.G. Rammensee, T. Friede, S. Stevanoviic, MHC ligands and peptide motifs: first listing, Immunogenetics 41 (1995) 178–228.
[31] G. Futaki, H. Kobayashi, K. Sato, M. Taneichi, M. Katagiri, Naturally processed HLA-DR9/DR53 (DRB1*0901/DRB4*0101)-bound peptides, Immunogenetics 42 (1995) 299–301.
[32] T. Tana, N. Kamikawaji, C.J. Savoie, T. Sudo, Y. Kinoshita, T. Sasazuki, An HLA-binding-motif-aided peptide epitope library: a novel library design for the screening of HLA-DR4-restricted antigenic peptides recognized by CD4+T cells, J. Hum. Genet. 43 (1998) 14–21.
[33] M. Hashimoto, T. Kinoshita, M. Yamasaki, H. Tanaka, T. Imanishi, H. Ihara, Y. Ichikawa, T. Fukunishi, Gene frequencies and haplotypic associations within the HLA region in 916 unrelated Japanese individuals, Tissue Antigens 44 (1994) 166–173.
[34] K. Mizumoto, S. Kimura, Y. Abe, M. Uehara, M. Katagiri, [Analysis of T cell epitopes on birch pollen allergen], Hokkaido Igaku Zasshi 72 (1997) 59–67.
[35] T. Hiroi, K. Fujihashi, J.R. McGhee, H. Kiyono, Polarized Th2 cytokine expression by both mucosal gamma delta and alpha beta T cells, Eur. J. Immunol. 25 (1995) 2743–2751.
[36] K.W. Beagley, J.H. Eldridge, H. Kiyono, M.P. Everson, W.J. Koopman, T. Honjo, J.R. McGhee, Recombinant murine IL-5 induces high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells, J. Immunol. 141 (1988) 2035–2042.
[37] K.W. Beagley, J.H. Eldridge, F. Lee, H. Kiyono, M.P. Everson, W.J. Koopman, T. Hirano, T. Kishimoto, J.R. McGhee, Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells, J. Exp. Med. 169 (1989) 2133–2148.
[38] T. Defrance, B. Vanbervliet, F. Briere, I. Durand, F. Rousset, J. Banchereau, Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A, J. Exp. Med. 175 (1992) 671–682.
[39] Y. Kurono, M. Suzuki, G. Mogi, M. Yamamoto, K. Fujihashi, J.R. McGhee, H. Kiyono, Effects of intranasal immunization on protective immunity against otitis media, Int. J. Pediatr. Otorhinolaryngol. 49 (Suppl 1) (1999) S227–S229.
[40] M. Marinaro, P.N. Boyaka, F.D. Finkelman, H. Kiyono, R.J. Jackson, E. Jirillo, J.R. McGhee, Oral but not parenteral interleukin (IL)-12 redirects T helper 2 (Th2)-type responses to an oral vaccine without altering mucosal IgA responses, J. Exp. Med. 185 (1997) 415–427.
[41] M.B. Joshi, A.A. Gam, R.A. Boykins, S. Kumar, J. Sacci, S.L. Hoffman, H.L. Nakhasi, R.T. Kenney, Immunogenicity of well-characterized synthetic *Plasmodium falciparum* multiple antigen peptide conjugates, Infect. Immun. 69 (2001) 4884–4890.
[42] S.K. Chai, P. Clavijo, J.P. Tam, F. Zavala, Immunogenic properties of multiple antigen peptide systems containing defined T and B epitopes, J. Immunol. 149 (1992) 2385–2390.