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Naturally processed measles virus peptide eluted from class II HLA-DRB1*03 recognized by T lymphocytes from human blood

Inna G. Ovsyannikova,[a] Kenneth L. Johnson,[b,c,d] Stephen Naylor,[b,c,1] David C. Muddiman,[c,d] and Gregory A. Poland[a,*]

[a] *Mayo Vaccine Research Group, Mayo Clinic and Foundation, Rochester, MN 55905, USA*
[b] *Biomedical Mass Spectrometry and Functional Proteomics Facility, Mayo Clinic and Foundation, Rochester, MN 55905, USA*
[c] *Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, MN 55905, USA*
[d] *W.M. Keck FT-ICR Mass Spectrometry Laboratory, Mayo Proteomics Research Center, Mayo Clinic and Foundation, Rochester, MN 55905, USA*

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Abstract

This is the first report of the direct identification of a HLA-DRB1*03 measles-derived peptide from measles virus infected EBV-transformed B cells. We purified HLA-DR3-peptide complexes from EBV-B cells infected with measles virus (Edmonston strain) and sequenced the HLA-DR3-peptides by mass spectrometry. A class II peptide, derived from a measles phosphoprotein, ASDVETAE-GGEIHELLRLQ (P1, residues 179–197), exhibited the capacity to stimulate peripheral blood mononuclear cells to proliferate. Our data provides direct evidence that the antigenic peptide of measles virus was processed by antigen-presenting cells, presented in the context of HLA class II molecules, and was recognized by peripheral blood T cells from healthy individuals previously immunized with measles vaccine. The approach described herein provides a useful methodology for the future identification of HLA-presented pathogen-derived epitopes using mass spectrometry. The study of cell-mediated immune responses to the measles-derived peptide in immune persons should provide significant insight into the design and development of new vaccines.

Keywords: Measles-derived peptide; HLA-DRB1*03; Measles virus; Mass spectrometry; Lymphocyte proliferation

Introduction

The World Health Organization has targeted measles for worldwide eradication, requiring an immunogenic vaccine for the genetically heterogeneous outbred population. Although well controlled by vaccination programs in industrialized countries, measles virus (MV) infection continues to be one of the major causes of childhood morbidity and mortality in developing countries (Gellin and Katz, 1994). The requirement for a cold chain (storage), the induction of low seroconversion rates in the presence of maternal antibodies, the vaccine failure rate, and the inability to use the vaccine in some immunocompromised conditions are the major drawbacks of the live-attenuated measles vaccine (El Kasmi et al., 2000; Albrecht et al., 1977). The limitations of the live vaccine combined with inadequate coverage in developing countries leads to approximately one million measles-related deaths annually (Jaye et al., 1998; Sabin, 1991). Thus, there is a need to develop alternative vaccines that are thermostable, safe, and designed to avoid recognition and neutralization by passive maternal antibodies (El Kasmi et al., 1999; Jaye et al., 1998). Such vaccines should induce long-lasting cell-mediated and humoral immune responses. For this reason, the development of a candidate peptide-based vaccine, based on immunologically relevant information on naturally processed and presented measles

* Corresponding author. Mayo Vaccine Research Group, Mayo Clinic, Guggenheim 611C, 200 1st Street S.W., Rochester, Minnesota 55905. Fax: +1-507-266-4716.

E-mail address: poland.gregory@mayo.edu (G.A. Poland).

[1] Present address: Beyond Genomics, 40 Bear Hill Road, Waltham, MA 02451.

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virus-derived peptides eluted from HLA class I and class II molecules, would have a significant impact.

Defining peptide epitopes recognized by $CD8^+$ and $CD4^+$ T lymphocytes involved in immune responses has generated tremendous interest (Germain, 1994a). We previously demonstrated that humoral immune responses to measles-encoded proteins are strongly associated with the human leukocyte antigen (HLA) class I and class II genes (Poland et al., 1999). In particular, HLA-DRB1*03 (DR3) alleles are significantly associated with measles vaccine seronegativity and play an important role in the immune response to MV (Poland et al., 2001a). Identification and comparison of the repertoire of measles-derived peptides that bind to class II HLA-DR3 molecules in poor and high responders to measles vaccine is important for designing effective vaccines against measles. The HLA class I and class II antigen-processing pathways play a critical role in the activation of measles-specific T lymphocytes by presenting peptide epitopes derived from viral proteins (Pamer, 1999). The HLA class II molecules bind and present exogenous measles antigens for recognition by $CD4^+$ helper T cells and play an important role in the immune response to measles (Germain, 1994b, 1995; Pamer, 1999). Alternatively, class II molecules can also use the endogenous pathway of measles virus antigen presentation (Nuchtern et al., 1990; Sekaly et al., 1988). Identification of such immunogenic measles epitopes, which are recognized by T and B lymphocytes, would advance peptide-based therapies and vaccine development (Poland et al., 2001b). However, a potential obstacle to the development of a peptide-based measles vaccine is the high degree of human HLA gene polymorphism (Doolan et al., 2000).

HLA molecules bind antigenic peptides and display them to T cell receptors on the surface of helper T cells (Garcia et al., 1999; Brown et al., 1988; Stern et al., 1994). Adoptive immune responses are therefore limited by the spectrum of immunogenic peptides displayed to T cells. Limitations in identifying class II peptides include the difficulty in detecting pathogen-derived peptides eluted from HLA class II–peptide complexes and the lack of knowledge regarding HLA class II presentation of measles virus peptides, as only a few human measles virus class I peptides and HLA class II restricted cytotoxic T lymphocytes (CTL) responses are described in the literature (Herberts et al., 2001; van Els et al., 2000; van Binnendijk et al., 1993; Jacobson et al., 1989). However, the rapid characterization of defined peptides that are critical to viral immunity, including measles, has been significantly enhanced by mass spectrometry (MS), which provides peptide sequence information at the femtomole level of sensitivity.

Although direct sequencing of naturally processed peptides bound to HLA class I and II molecules by liquid chromatography mass spectrometry (LC-MS) is well-established (Dongre et al., 2001; de Jong, 1998), identification of pathogen-derived peptides presents a formidable challenge due to the diverse range of low-abundance peptides presented by HLA molecules. Strategies to reduce the complexity of the mixture prior to introduction into the mass spectrometer have often relied on multiple steps of reversed-phase (RP) liquid chromatography. However, this approach does not effectively increase the peak capacity because the separation mechanisms of each RP chromatography step are not orthogonal.

We have adopted an approach, developed in the field of proteomics, to resolve the profound biological complexity presented in these investigations. The approach is based on two truly orthogonal separation techniques, namely, (1) strong cation exchange (SCX) chromatography, which separates peptides based on their charge; and (2) nano-RP high-performance liquid chromatography, which uses hydrophobicity (Link et al., 1999; Washburn et al., 2001). This fully automated, multidimensional chromatography-MS approach affords a geometric increase in the overall peak capacity that dramatically increases the effective dynamic range and the number of peptides that can be dissociated (sequenced using data-dependent tandem-MS) for any given sample.

An overview of the methodology we developed for identifying MHC class II peptides originating from measles virus is shown in Fig. 1. This methodology provides a powerful tool for the identification of pathogen-derived HLA class II peptides that in turn can be evaluated as potential subunit vaccine candidates. We report here for the first time that naturally processed measles phosphoprotein (P)-derived peptide was isolated and sequenced from class II HLA-DR3 molecules of measles virus infected EBV-transformed B (EBV-B) cell lines by mass spectrometry.

Results

Identification of the measles-specific HLA-DR3 peptides by 2D nLC tandem-MS

An aliquot representing 25% of the peptide extract from measles virus infected cells was subjected to two-dimensional nLC, data-dependent tandem-MS and acquired a total of 1371 tandem mass spectra from 10 SCX fractions. Peptide sequences were identified by searching the spectra against a subset of the NR database from NCBI using SEQUEST software (Eng et al., 1994). Search results were initially filtered on the basis of their cross-correlation score ($X_{\mathrm{Corr}} > 2$). From the 1371 tandem mass spectra acquired, 276 spectra met the search criteria, of which only one spectrum returned a search result for a measles virus peptide. The tandem mass spectrum of a triply charged precursor with a $m/z = 689.69$ ($[M + H^+]^+ = 2067.0_5$), eluting in the 40 mM KCl SCX fraction, returned a SEQUEST search result where the two top-ranked sequences were peptides from multiple database entries for phosphoproteins from the measles virus (Fig. 2A). The two candidate sequences, ASDVETAEGGEIHELLRLQ (designated MV-

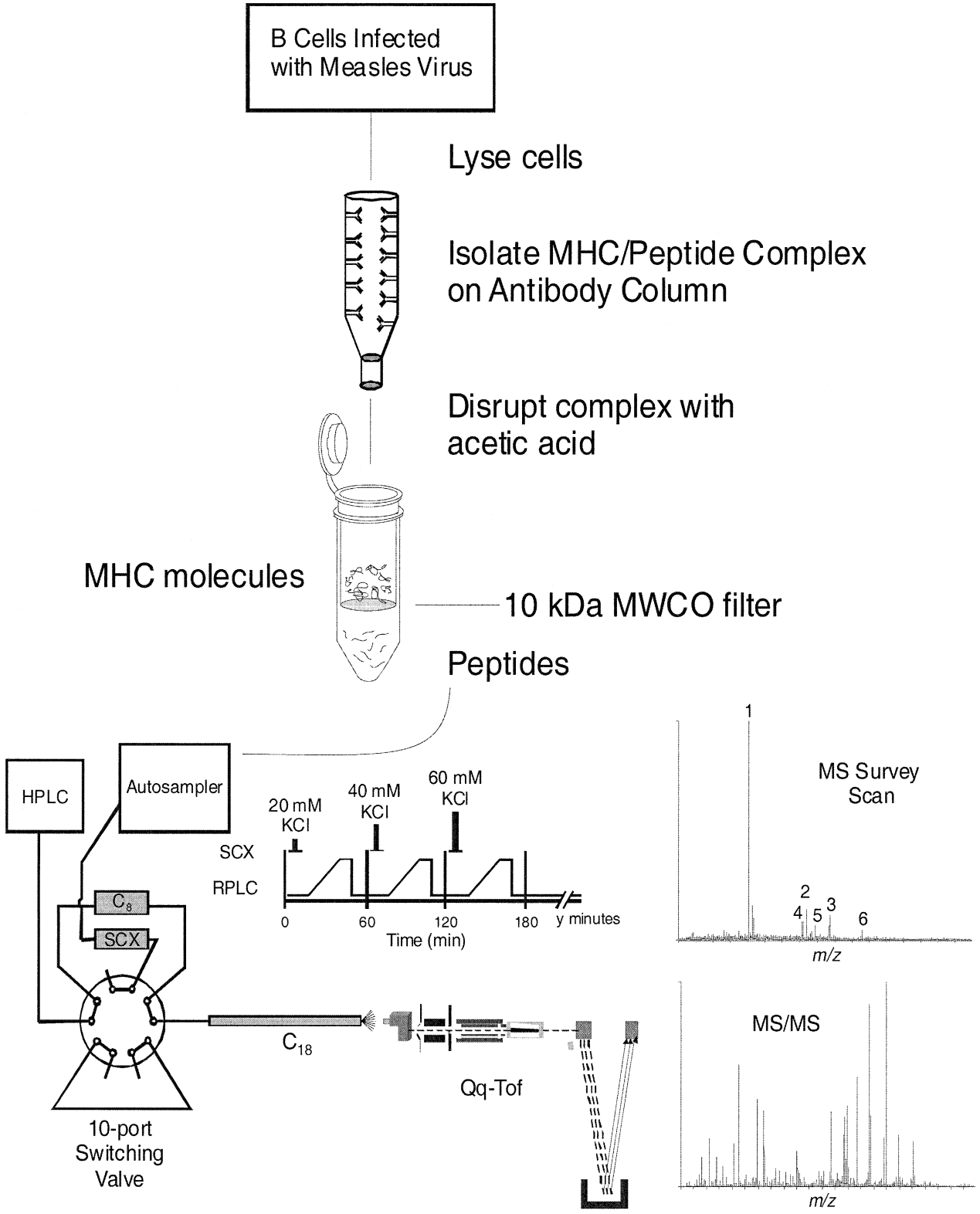

Fig. 1. Overview of the analytical method for isolating and sequencing MHC class II peptides. B cells infected with measles virus are lysed and MHC molecule/peptide complexes are isolated on an antibody column. Dissociated peptides were loaded onto an automated 2D-LC-MS system. Peptides were eluted from the SCX column by salt steps introduced by the autosampler. Data-dependent MS/MS experiments were conducted during the subsequent reversed-phase nano-LC separations.

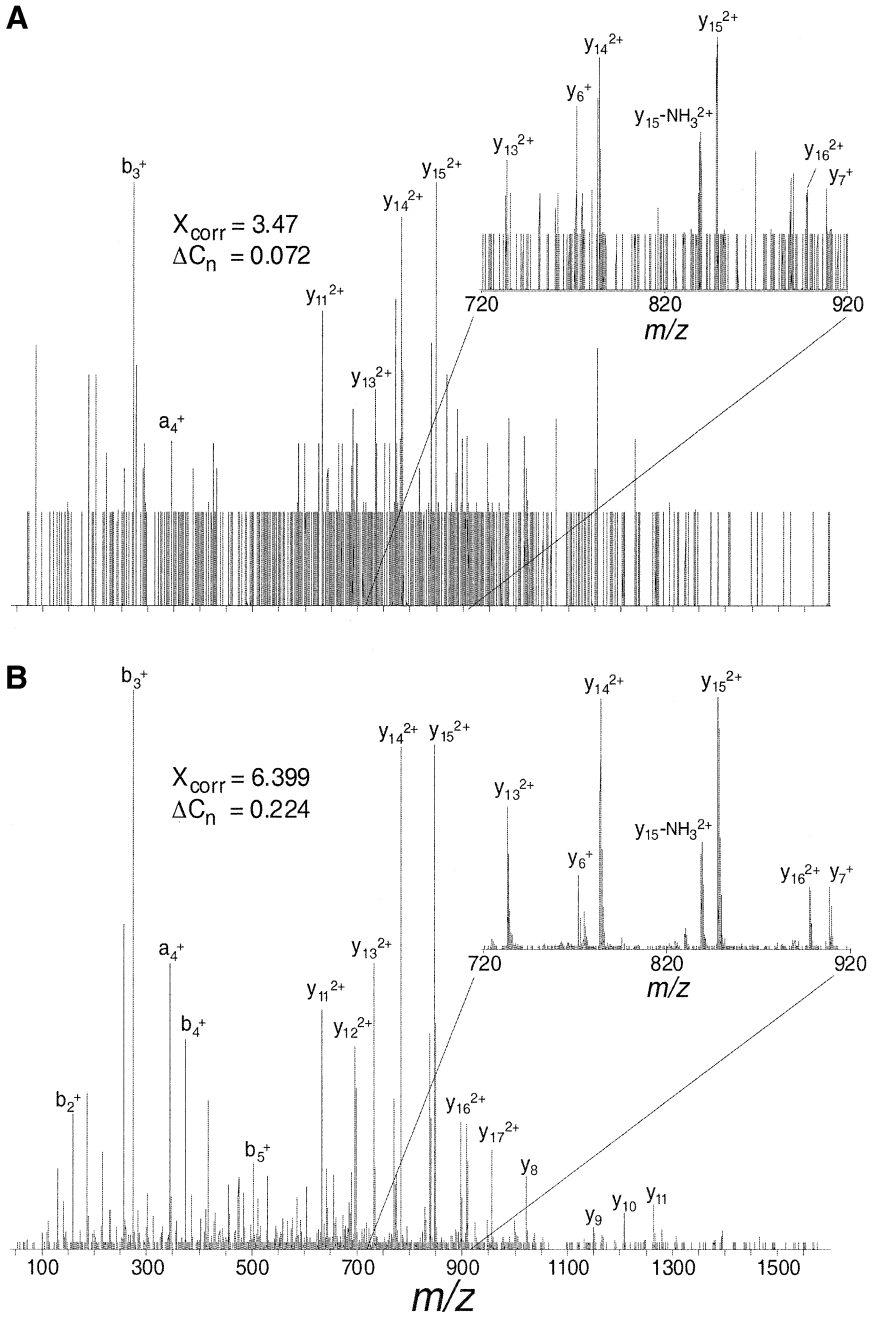

Fig. 2. Tandem mass spectra of *m/z* = 689.7 obtained from the 40 mM SCX fraction with their corresponding SEQUEST scores. (A) Naturally processed peptide (inset shows an expansion of the *m/z* range 720 to 920). (B) Synthetic peptide ASDVETAEGGEIHELLRLQ (inset shows an expansion of the *m/z* range 720 to 920).

P1) and ASDVETAEGGEIHKLLRLQ (designated MV-P2), differ only by one amino acid, a Glu (E) versus Lys (K) at position 192.

Although the search statistics did not conclusively rule out the MV-P2 sequence, the difference between the two candidate sequences, MV-P1 and MV-P2, is a nonconservative amino acid change that can readily be distinguished solely by molecular weight as the peptide ion mass differs by 1 Da. The experimental monoisotopic mass for the naturally processed peptide was $[M + H^+]^+ = 2067.05$, while the theoretical values for MV-P1 and MV-P2 are 2067.03 and 2066.09, respectively, clearly in agreement with MV-P1 (~10 ppm mass error vs nearly 500 ppm for MV-P2). Several of the expected product ions from synthetic MV-P2 are also one mass unit lower than the observed product ions in the naturally processed spectrum (data not shown).

The tandem mass spectra of synthetic MV-P1 (Fig. 2B) relative to the naturally processed peptide (Fig. 2A) are quite similar. Although product ions in the tandem mass

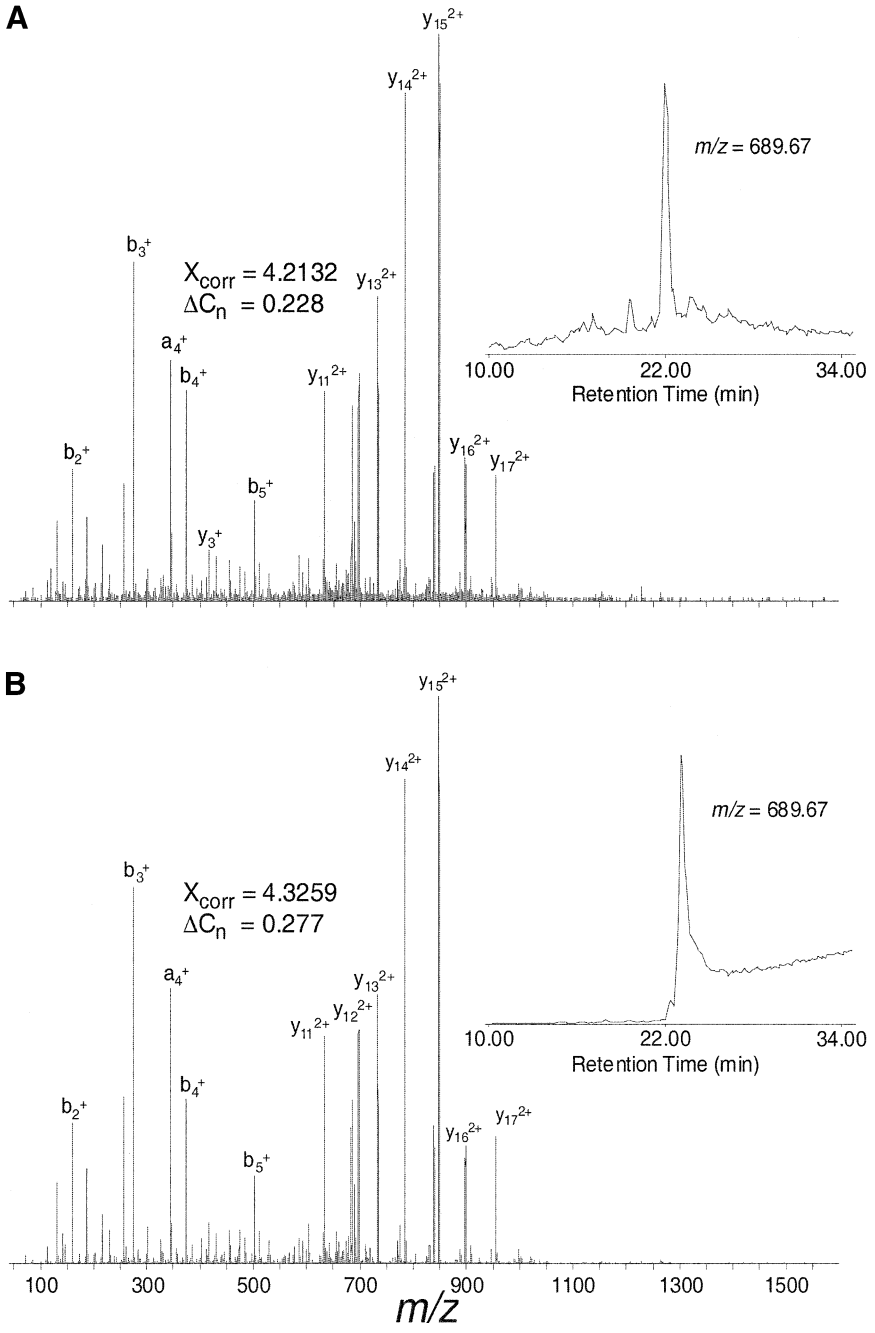

Fig. 3. Tandem mass spectra of *m/z* 689.7 from (A) naturally processed peptide with targeted data-dependent analysis and increased loading as compared to the data shown in Fig. 2a (inset shows the selected ion current for $m/z = 689.67 \pm 0.5$ over a 15-min RP retention time window); (B) naturally processed peptide spiked with 500 fmol of the synthetic peptide (inset shows the selected ion current for $m/z = 689.67 \pm 0.5$ over a 15-min RP retention time window). The peak tailing in Fig. 3b clearly indicates we have overloaded the column in the standard additions experiment; however, the retention times in Figs. 3A and B are still within 5% of each other.

spectrum from the naturally processed peptide (Fig. 2A) are only marginally more intense than noise, a series of doubly charged *y* product ions ranging from y_{11} to y_{16}, as well as the singly charged b_2, b_3, and a_4 product ions, are observed for both the naturally processed and the synthetic peptides, which resulted in significant cross-correlation (X_{corr}) and ΔC_n scores (Figs. 2A and B) (Smith et al., 2002; Eng et al., 1994).

To be prudent, we carried out additional measurements to ensure confident identification of the naturally processed MHC class II peptide. First, we used the synthetic peptide to optimize the collision energy for the tandem MS experiments (optimized collision voltage of 24 V shown in Figs. 3A and B vs 26.8 V for the data shown in Figs. 2A and B). Second, we adopted a more focused data-dependent analysis where $m/z = 698.7$ was selected as a priority precursor

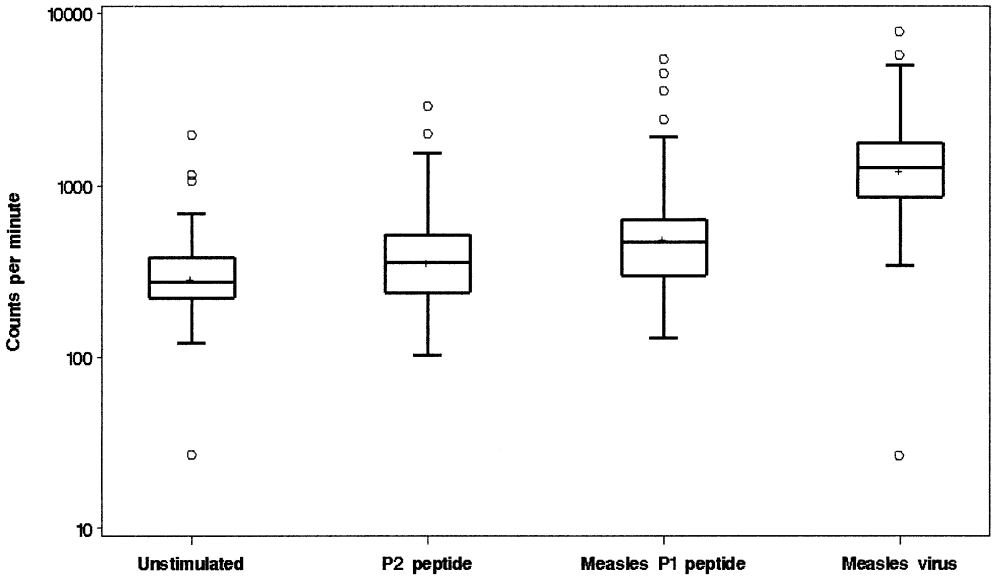

Fig. 4. Box plots of counts per minute (cpm) by lymphoproliferative responses. Values are presented on a log scale. Top and bottom of boxes represent the third and first quartiles, respectively. Middle line represents median, plus sign represents mean, and vertical lines represent values falling within 1.5 times the interquartile range to either side of the first and third quartile. Circles represent outliers falling outside of the vertical lines.

ion and the survey scan was restricted to $m/z = 650$ to 720. Although this strategy does not enhance the minimum level of detection, it acts as an additional dimension of separation in the gas phase that focuses data-dependent acquisition on fewer potential precursor peptides at the expense of having to carry out multiple runs (Spahr et al., 2002). Third, we used an aliquot representing 50% of the total extract in an attempt to improve the signal-to-noise ratio of the product–ion spectrum. Fourth, we designed a standard addition experiment to determine if the synthetic peptide coeluted in the same SCX fraction and subsequently had the same RP retention time as the naturally processed sample (i.e., an increase in the signal-to-noise for the spiked sample).

We analyzed an aliquot representing 50% of the total extract by two-dimensional nLC tandem-MS after replacing the SCX, C_8, and C_{18} columns with new columns that had not seen any synthetic MV-P1 peptide or naturally processed peptide extracts. These experiments again yielded a tandem mass spectrum for *m/z* 689.65 (Fig. 3A) eluting in the 40 mM KCl fraction. A small amount of this *m/z* was also detected, at the same reversed-phase retention time, in the 60 mM KCl fraction. For the standard addition experiment, 500 fmol of synthetic MV-P1 was spiked, prior to the initial desalting step described under Materials and methods, into the remaining aliquot of peptide extract representing 17% of the total. Synthetic MV-P1 eluted predominantly in the 40 mM SCX fraction (65% of total response) with 21% of the total response being detected in the 20 mM KCl fraction and 14% in the 60 mM KCl fraction. The tandem mass spectrum of the coeluting synthetic MV-P1 and naturally processed MV-P1 from the 40 mM KCl fraction is shown in Fig. 3B. Clearly, the synthetic peptide behaves identically to the naturally processed peptide identified in earlier experiments (Fig. 2A). Thus, we conclude that we have identified the naturally processed peptide as being ASDVETAEGGEIHELLRLQ (MV-P1) from the measles virus phosphoprotein.

Finally, by comparing the relative responses from the standard addition experiment (Fig. 3B) to the naturally processed sample (Fig. 3A), we estimate that the tandem MS spectrum represents approximately 20 fmol of the naturally processed peptide (Fig. 3A). Relative to responses observed for other peptides from endogenous proteins, the MV-P1 peptide is a minor epitope, where the more abundant endogenous peptides were observed with 100-fold higher MS responses than observed for MV-P1.

Proliferative response of vaccinated donors to measles P1 and P2 peptides

We examined recognition of these measles-derived peptides by peripheral blood T cells from 95 healthy subjects previously immunized with measles-mumps-rubella-II (MMR-II) vaccine as a means of determining the immunologic relevance of this peptide. PBMC from vaccinated subjects were responsive to synthetic P1 and P2 peptides in vitro. The results revealed large interindividual variation among 95 tested subjects, but we observed little variability between experiments on the same subject. Fig. 4 shows the distribution of counts per minute (cpm) in lymphoproliferative assays. Using a cutoff value for significant lymphoproliferative responses ($SI \geq 3$), the stimulatory responses could be grouped into the following patterns of response. The median cpm value was lower for unstimulated cells (cpm = 274) than for MV vaccine (cpm = 1277, $P <$

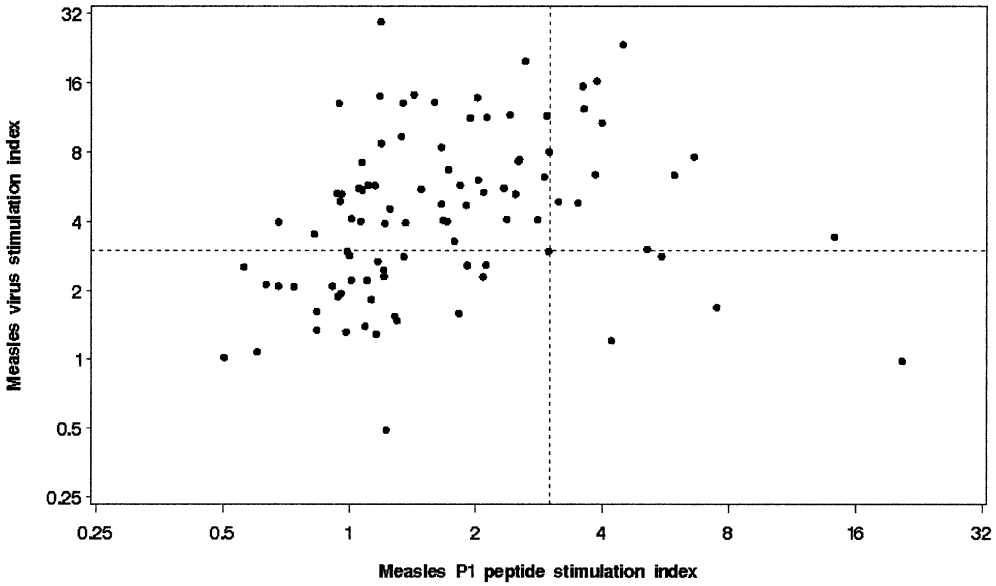

Fig. 5. Plot of MV stimulation indices with measles P1 peptide stimulation indices. Values are graphed on a log scale. Dashed lines indicate proliferative responsiveness cut point of 3.0. Spearman rank correlation coefficient is 0.38 ($P < 0.001$), sensitivity = 0.20, specificity = 0.89.

0.001), measles-derived P1 (cpm = 472, $P < 0.001$), and P2 (cpm = 359, $P < 0.001$) stimulated cells.

Measles virus stimulation indices (median 4.1, range 0.5–29.1) were generally higher than measles P1 peptide (median 1.4, range 0.5–20.3) or P2 peptide stimulation indices (median 1.2, range 0.5–16.2). Figs. 5 and 6 indicate modest but positive correlations of MV-stimulated lymphoproliferative responses (SI) with P1 and P2 SIs (Spearman correlation coefficients = 0.38 and 0.21, respectively) across all subjects. Sixty of the 95 subjects (63%) had MV stimulation indices greater than 3.0, indicating that measles vaccine virus contains multiple T cell epitopes. Comparatively, measles-derived P1 and P2 peptides were recognized in 17 and 5% of the subjects, respectively, thereby suggesting a higher frequency of P1-specific T cells in subjects after measles immunization. Among the 60 subjects who responded to the MV, 12 also responded to the P1 peptide (sensitivity = 20%) and three responded to the P2 peptide (sensitivity = 5%). We saw little or no proliferation in healthy subjects who were immunized with MMR-II vac-

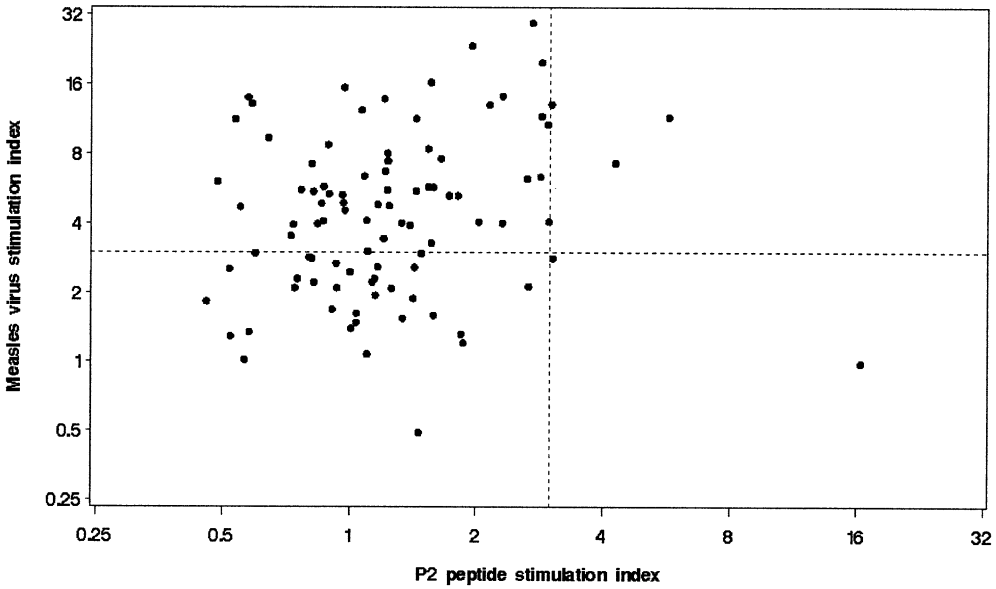

Fig. 6. Plot of MV stimulation indices with measles P2 peptide stimulation indices. Values are graphed on a log scale. Dashed lines indicate proliferative responsiveness cut point of 3.0. Spearman rank correlation coefficient is 0.21 ($P = 0.04$), sensitivity = 0.05, specificity = 0.94.

cine to randomly chosen measles fusion (F) peptide from the MV proteome (data not shown). Thus, the lymphoproliferative response of the vaccinated subjects to measles naturally processed P1 peptide may be of further interest in studies to investigate induction of protective immunity to measles.

Discussion

The identification and characterization of antigenic epitopes of infectious pathogens by $CD4^+$ T cells is of major interest (Peakman et al., 1999; Germain, 1994a; Germain and Hendrix, 1991). In our study, we identified a HLA class II naturally processed peptide derived from MV phosphoprotein. The amino acid sequence of P1 peptide (ASDVETAEGGEIHELLRLQ) obtained from direct sequencing by nLC/MS/MS was concordant with the measles viral genome.

Measles is a negative-strand RNA virus. Measles virus P gene of *Paramyxoviruses* encodes three proteins: P polypeptide and two nonstructural gene products, C and V polypeptides, which encode virulence functions in vivo (Patterson et al., 2000). The P gene encodes a heavily phosphorylated protein (60 kDa), which, in association with the polymerase (L) protein, is required for transcription and replication of the ribonucleoprotein complex (Griffin and Bellini, 1996). In addition, P protein also acts as a chaperone that interacts with and regulates the cellular localization of nucleocapsid (N) protein and may assist in N assembly (Griffin and Bellini, 1996; Horikami and Moyer, 1995). Animals challenged with recombinant virus expressing the H, N, or F measles structural protein were protected against measles encephalitis, whereas matrix (M) or P protein immunization provided only partial protection (Brinckmann et al., 1991).

The significance of our results resides in a technique capable of identifying naturally processed pathogen-derived peptides eluted from the open peptide binding groove of class II HLA-DR molecules and the potential use of this technique in directed vaccine development. Furthermore, we have established the immunologic relevance of these peptides by demonstrating their ability to induce recall immunity to measles in a lymphoproliferation assay among HLA discordant subjects. Previous investigations of in vitro PBMC proliferative responses to overlapping measles peptides were difficult, and often a short-term preculture with MV antigen and/or development of MV-specific T cell lines and T cell clones were needed to visualize significant proliferative response (Marttila et al., 1999). We detected responses to a single P1 epitope, representing residues 179–197, in 17% of subjects without prior amplification of specific cells among HLA discordant subjects. The measles-derived P1 peptide is antigenic, as assessed by its capacity to be recognized by PBMC isolated from subjects previously immunized with measles vaccine. Since we obtained PBMC from subjects with unknown HLA types (i.e., many were unlikely to be DR3 positive), it is likely that the number of true peptide responders is underestimated. We might, in fact, expect a low lymphoproliferative response among DR3 subjects, as the stimulating peptide is a "non-responder" peptide derived from DR3-positive subject. These data provide direct evidence that MV antigenic peptides were processed and could bind to HLA class II molecules. This information can only be obtained by direct elution from class II HLA molecules isolated from APC.

Isolation and identification of naturally processed and presented peptides greatly accelerates our ability to understand mechanisms of immunogenicity and further illustrates the importance of immunogenetics. It is conceivable that vaccine nonresponders present a different spectrum of peptides to the immune system compared to vaccine responders. If so, the importance of HLA restriction in the immune response becomes primary in designing strategies to induce protective immune responses. Such an understanding also suggests an important approach to the directed design of new vaccines. It may be possible to design a vaccine that is a "cocktail" of peptides that induces protective immune responses across the spectrum of a population's HLA variability. The current limitation to this approach is the empiric and inefficient process of identifying which peptides are important in inducing a protective immunity and how they are HLA-restricted, and demonstrating the immunologic relevance of such peptides. Importantly, our results suggest an important advance, as our process could be applied to directed development of vaccines for any disease process where stimulating antigens can be identified. For example, our approach may elucidate which tumor-specific peptides are presented to the immune system in a successful response to a given cancer. Similarly, new and safer vaccines against infectious diseases can be designed. For example, a peptide vaccine that induces immunity to the variola virus (smallpox) might allow universal immunization as opposed to the limitations imposed by a live, albeit attenuated, whole virus vaccine.

Our approach also has limitations. Given current technology, isolating and identifying HLA-derived class II peptides is similar to searching for the proverbial needle in the haystack. Peptides of lower abundance are less likely to be identified than peptides of higher abundance. Class II peptides are more difficult to identify than class I peptides, due to the open ends of the peptide binding groove, allowing both longer and more promiscuous peptides to be bound by the HLA molecule. We are currently developing hybrid Fourier-transform ion cyclotron resonance mass spectrometry instrumentation to afford significantly higher peak capacity (resolving power) and the ability to detect peptides in extremely low abundance. In the present study, we have used a lymphocyte proliferation assay as a measure of recall cellular immunity to "screen" and determine the immunologic relevance of measles-derived peptides. However, the generation of short-term and long-term measles peptide-

specific T cell clones or cell lines and characterization of HLA-DR3-restricted $CD4^+$ T cell epitopes of measles antigens is very important for determining the functional specificity of the identified peptide. This is a topic for a separate report.

In conclusion, we report the first class II measles-derived peptide directly isolated from the class II HLA peptide binding groove of the DR3 molecule in humans. A critical contribution to this accomplishment was the development of a comprehensive, integrated, and automated analytical strategy that allowed for the identification of this peptide. Mass spectrometry will be the cornerstone technology in directed vaccine development by allowing the identification of naturally processed pathogen-derived peptides for the rational development of new peptide-based vaccines.

Materials and methods

Donor cell preparation

We generated an EBV-B cell line from peripheral blood mononuclear cells (PBMC) of an HLA-DR3 homozygous patient using 1×10^7 PBMC and the B95-8 strain of EBV (American Type Culture Collection, Manassas, VA) in RPMI medium containing 1 μg/ml cyclosporin A (Neitzel, 1986). We obtained a heparinized venous blood (20 U/ml heparin) sample from a single EBV-seronegative subject (K.E., 16-year-old female, DRB1*0301, A*1/3, B*8/44, C*7), who had been immunized with two doses of live-attenuated measles vaccine (Attenuvax, Merck, West Point, PA). The subject had no previous history of measles infection. The circulating MV-specific IgG antibody titer in the subject's sera was determined by and IgG whole virus specific EIA (MeasleELISA, BioWhittaker, Walkersville, MD). The subject was characterized as a measles vaccine responder (EIA MV antibody titer = 2.43 U/ml). B cells were subcultured four to six times before being used as antigen-presenting cells (APC) and were routinely monitored for HLA-DR expression by flow cytometry.

Human subjects

Study participants included 95 healthy residents of Olmsted County, MN, aged 11 to 18 years. The subjects' medical records documented that each subject had been previously immunized with two doses of MMR-II vaccine (Merck Research) containing the Edmonston strain of MV (tissue culture infective dose, $TCID_{50} \geq 1000$) dose. All subjects resided in a geographic area where no wild-type MV had circulated in the community during the subjects' lifetimes. The Institutional Review Board (IRB) of the Mayo Clinic granted approval for the study, and peripheral blood samples were drawn after informed consent was obtained from each subject. Mononuclear leukocytes were isolated by Ficoll–Hypaque (Amersham) density gradient centrifugation.

Cell cultures and virus infection

We grew the Edmonston vaccine strain of measles in Vero cells, in Dulbecco's modified Eagle's medium, supplemented with 5% fetal calf serum (FCS) (virus stocks of 2.2×10^7 PFU/ml). Subsequently, EBV-B cells were infected with live MV at a multiplicity of infection (m.o.i) of 1 PFU/cell for 1 h and maintained for 36–48 h at 37°C in RPMI-1640 containing 2% FCS (Life Technologies, Gaithersburg, MD). Equally sized batches of MV-infected and uninfected cells were washed in PBS, pelleted, and stored at-80°C. We monitored the infection of cells by flow cytometry using purified monoclonal antibody (mAb) specific for MV H protein tagged with FITC (Virostat, Portland, ME) (Naniche et al., 1993) (data not shown).

Immunoaffinity purification of HLA-DR3 molecules and associated peptides

An overview of our methodological strategy has been previously published (Poland et al., 2001b). We used the same number of uninfected and MV-infected cells for HLA-DR-peptide complex purification. DR3-bound peptides were isolated from immunoaffinity purified class II molecules as previously described (Ovsyannikova et al., 2000; Kirschmann et al., 1995). Briefly, 8 g cell pellets consisting of either infected or uninfected cells were lysed in 1% CHAPS, 150 mM NaCl, 20 mM Tris–HCl, pH 8.0, and 1 mM Pefabloc SC (Boehringer Mannheim GmbH, Germany). The lysates were centrifuged at 100,000 *g* for 2 h and the HLA-peptide complexes were immunoprecipitated from the supernatants using an anti-HLA-DR mAb specific for a HLA-DR monomorphic epitope (L227, IgG1) (Lampson and Levy, 1980) covalently linked to CNBr-activated Sepharose 4B beads (Sigma). The column was washed sequentially with five separate washings, first using 10 column volumes of lysis buffer; five column volumes of 0.1% deoxycholic acid (Boehringer Mannheim GmbH), 20 mM Tris, pH 7.4; five column volumes of 20 mM Tris, 500 mM NaCl, pH 7.4; five column volumes of 20 mM Tris, 150 mM NaCl, pH 7.4, then using five column volumes of 20 mM Tris, pH 7.4. After these series of wash steps, the HLA-DR-peptide complexes were eluted from the affinity column (pH 11.5) with 0.1% deoxycholic acid and 50 mM glycine. We neutralized the eluates with 2 M glycine and concentrated them in a Centricon-10 (Amicon, Beverly, MA) before a second round of precipitation by 14% acetic acid to dissociate any bound peptides from DR3 molecules. HLA-DR3 molecules were more than 99% pure as assessed by SDS–PAGE. We determined protein concentration by BCA assay (Pierce, Rockford, IL). The peptides were concentrated in a spin vacuum to 100 μL aliquots (1×10^9 cells) and stored at −80°C for later analysis by MS.

Peptide sequencing methodology

HLA class II restricted peptides were sequenced using automated two dimensional liquid chromatography (strong cation exchange followed by nanoscale reversed phase, SCX, and nLC, respectively) coupled via nanoelectrospray, to a Micromass Q-Tof-2 tandem mass spectrometer (Micromass Ltd., Manchester, UK).

Prior to SCX, the peptide pool was desalted using a reversed-phase microcolumn (Peptide Trap, Michrom BioResources Inc., Auburn, CA). Desalted peptides, in SCX mobile phase A, were loaded on a 300 μm i.d. by 5-mm-long column of Polysulfoethyl A (PolyLC, Inc., The Nest Group, Southborough, MA). Peptides were step-eluted from the SCX column using KCl concentrations of 20, 40, 60, 80, 100, 150, 200, 250, and 500 mM and were reconcentrated on a precolumn before being chromatographed in the reversed-phase dimension. The precolumn was 300 μm i.d. by 5 mm long (LC Packings, San Francisco, CA) packed with Magic C_8 (5 μm, 300 Å) (Michrom BioResources). SCX mobile phase A was water/acetonitrile/*n*-propanol (95/4/1 v/v/v), containing 10 mM potassium phosphate, pH = 3.1.

We performed nano scale LC in a 75 μm i.d. PicoFrit column (New Objective, Woburn, MA) packed with 5.5 cm of Magic C_{18} (5 μm, 200 Å) (Michrom BioResources Inc.). Reverse mobile phase A was water/acetonitrile/ *n*-propanol (98/1/1 v/v/v) with a 0.2% overall concentration of formic acid. Reverse mobile phase B was acetonitrile/*n*-propanol/water (80/10/10 v/v/v) containing 0.2% formic acid overall. An LC pumping system, operated at 30 μL/min and split to 300 nL/min just prior to the switching valve, was used to generate a mobile phase gradient from 0 to 50% through the reversed-phase nLC column after each salt elution step.

We conducted tandem-MS experiments on precursor ions from doubly, triply, or quadruply charged ions within the *m/z* range of 450–1300; the collision energies were automatically selected as a function of *m/z* and charge (unless noted otherwise in the text) using argon as the collision target. Tandem mass spectra were searched, using SEQUEST software (ThermoFinnigan, San Jose, CA), against the combined subset of human, bovine, and measles proteins from the NR database (available February 2002 from ftp://ftp.ncbi.nih.gov/blast/db/nr) (Eng et al., 1994).

Synthetic peptides

Identified peptides were subsequently synthesized by the Mayo Protein Core Facility (Rochester, MN) using *N*-(9-fluorenyl)methoxycarbonyl protection chemistry and carbodiimide/*N*-hydroxybenzotriazole activation on a MPS 396 Multiple Peptide Synthesizer (Advanced Chemtech, Louisville, KY). We purified each peptide by RP HPLC and verified by mass spectrometry and amino acid (aa) analysis.

The following three peptides were used: (1) MV-derived naturally processed 19 aa P1 peptide of the measles P protein, ASDVETAEGGEIH**E**LLRLQ; (2) MV-P2 peptide, ASDVETAEGGEIH**K**LLRLQ;(3) MV-F control peptide of the MV fusion protein, PLRHQATTASSTKP, randomly chosen from MV F glycoprotein. Measles F control peptide was chosen for this study because of the established importance of measles F protein in cell-mediated immune response. In addition, Bakouche et al. show that the F protein of MV is a potent T cell antigen (Bakouche et al., 1987). The MV sequence corresponds to the Edmonston strain (Parks et al., 2001).

T cell proliferation assay

We tested three measles-derived peptides (P1, P2, and F) for the capacity to induce recall peptide-specific proliferative responses. PBMC (2×10^5) were incubated in medium (RPMI-1640 supplemented with 5% autologous sera, penicillin, 100 U/ml, 2-mercaptoethanol, and sodium pyruvate) alone or in the presence of phytohemagglutinin (PHA, 5 μg/ml) to assess cell vitality, or in the presence of measles-synthetic peptides in a concentration of 20 μg/ml, or live-attenuated MV (50 PFU/well) (Attenuvax, Merck). Cultures were incubated in a total volume of 200 μl for 3 days (37°C, 5% CO_2) and pulsed during the last 18 h with tritiated thymidine ^{3}H (1 μCi/well). We then harvested cells onto glass fiber filters, using a 96-well harvesting system (Skatron Instruments, Norway). The amount of incorporated radioactivity was determined by a liquid scintillation counter (Packard Instrument Co., Boston, MA) and the results were expressed as SIs. We calculated the SI as the ratio of mean cpm of triplicate wells of peptide stimulated to mean cpm of unstimulated control wells. Stimulation indices of $\geq$3 were considered to represent significant responses (Bautista-López et al., 2000; Marttila et al., 1999). We used six replicates of cpm values for unstimulated cells; three replicates each were used for T cells stimulated with MV-P1, MV-P2, MV-F, and live-attenuated MV vaccine. For each subject, median cpm was calculated for unstimulated cells, as well as for cells stimulated with MV-P1, MV-P2, and MV. These median values are used in all subsequent comparisons. Stimulation indices were calculated for P1, P2, F peptides, and MV using the median of the six unstimulated cpm values as the denominator.

Statistical analysis

For descriptive analyses, we used medians and ranges for continuous variables and frequencies for categorical variables. We compared cpm for MV-stimulated, MV-P1-stimulated and MV-P2-stimulated cells with unstimulated cells using Wilcoxon signed rank tests. Associations between the continuously distributed stimulation indices for MV-P1, MV-P2 peptides, and MV were determined using Spearman rank correlation coefficients. Stimulation indices were subsequently dichotomized into positive or negative using a cut

point of 3.0. We then compared MV positivity with MV-P1 and MV-P2 positivity using estimates of sensitivity.

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