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Characterizing immunodominant and protective influenza hemagglutinin epitopes by functional activity and relative binding to major histocompatibility complex class II sites

In the present study the analysis of functional activity and major histocompatibility complex (MHC) binding of two adjacent MHC class II-restricted epitopes, located in the C-terminal 306–329 region of human influenza A virus hemagglutinin 1 subunit (HA1) conserved with subtype sequences and not affected by antigenic drift, was undertaken to explore the hierarchy of local immnodominance. The functional activity of two T cell hybridomas of the memory/effector Th1 phenotype in combination with *in vivo* immunization studies provided a good tool for investigating the functional characteristics of the T cell resonse. The *in vitro* binding assays performed with a series of overlapping, N-terminal biotinylated peptides covering the 306–341 sequence enabled us to compare the relative binding efficiency of peptides, comprising two distinct epitopes of this region, to I-E^d expressed on living antigen-presenting cells. Our studies revealed that (i) immunization of BALB/c mice with the 306–329 H1 or H2 peptides resulted in the activation and proliferation of T cells recognizing both the 306–318 and the 317–329 epitopes, while the 306–329 H3 peptide elicits predomonantly 306–318-specific T cells, (ii) the 317–329 HA1 epitope of the H1 and H2 but not the H3 sequence is recognized by T cells and is available for recognition not only in the 317–329 peptide but also in the extended 306–329 or 306–341 peptides, (iii) the 306–318 and the 317–329 hemagglutinin peptides encompassing the H1, H2 but not the H3 sequence bind with an apparently similar affinity to and therefore compete for I-E^d binding sites, and (iv) the 317–341, the 317–329 peptides and their truncated analogs show subtype-dependent differences in MHC binding and those with lower binding capacity represent the H3 subtype sequences. These results demonstrate that differences in the binding capacity of peptides comprising two non-overlapping epitopes located in the C-terminal 306–329 region of HA1 of all three subtype-specific sequences to MHC class II provide a rationale for the local and also for the previously observed *in vivo* immunodominance of the 306–318 region over the 317–329 epitope in the H3 but not in the H1 or H2 sequences. In good correlation with the results of the binding and functional inhibition assays, these data demonstrate that in the H1 and H2 subtypes both regions are available for T cell recognition, they compete for the same restriction element with an appearently similar binding efficiency and, therefore, function as co-dominant epitopes. Due to the stabilizing effect of the fusion peptide, peptides comprising the 306–341 or 317–341 H1 sequences are highly immunogenic and elicit a protective immune response which involves the production of antibodies and interleukin-2 and tumor necrosis factor producing effector Th1 cells both directed against the 317–329 region. Based on the similarity of the I-E^d and HLA-DR1 peptide binding grooves and motifs, these results suggest that amino acid substitutions inserted to the H3 subtype sequence during viral evolution can modify the relative MHC binding capacity and invert the local hierarchy of immunodominance of two closely situated epitopes that are able to bind to the same MHC class II molecule.

[I 16269]

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Received September 24, 1996; in revised form August 18, 1997; accepted September 11, 1997.

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Abbreviations: HA: Hemagglutinin **HA0:** Immature hemagglutinin **HA1:** Subunit 1 of hemagglutinin **HA2:** Subunit 2 of hemagglutinin

Key words: Immunodominance / Major histocompatibility complex class II / Peptide binding / Influenza hemagglutinin

1 Introduction

Complex viral proteins carry multiple potential T cell epitopes but the response is focused to only a few immunodominant determinants. The factors dictating immunodominance include peptide affinity to MHC, antigen processing and the repertoire of T cells [1, 2]. In viral infection, demonstrated primarily by cytotoxic T cells, immunodominant epitopes elicit T cells detected during the course of natural infection, while subdominant epitopes evoke little or no response; however, the T cells activated by peptide immunization recognize virus-infected cells [3–5]. This type of limited diversity in specific T cell responses may select for pathogens that carry mutations in the dominant T cell epitopes and, therefore, may support escape from immune control [6, 7].

0014-2980/97/1212-3105$17.50+.50/0

The membrane protein hemagglutinin (HA) of human influenza A viruses is the major target of neutralizing antibody (Ab) and helper T (Th) cell responses (reviewed in [8–10]). A majority of $CD4^+$ memory T cells elicited by viral infection recognize HA1 regions identified as major Ab binding sites and their recognition is influenced by amino acid substitutions generated by antigenic drift [8, 11, 12]. The major HA1 site of the H3 subtype, recognized by Th cells in BALB/c mice, is covered by the 177–199 sequence comprising both I-A^d- and I-E^d-restricted epitopes [11, 12]. The T cell response against the H1 subtype focuses to three major regions located within the 110–120, 126–138 and 303–313 sequences [9, 13, 14]. In contrast to these variant HA1 regions, the C-terminal 24 amino acids of HA1 are characterized by subtype-specific but conserved sequences within the H1, H2 and H3 subtypes [15, 16]. BALB/c mice are high responders to the 306–329 H3 peptide and the peptide-specific response is directed against the 306–318 region, demonstrating its intrapeptide dominance over the C-terminal fragment [17, 18].

The H3N2 subtypes of human influenza A viruses have been common pathogens of recently occurring epidemics since 1968 [19–21] and the 306–329 sequence of H3 HA1 was also demonstrated to be immunodominant in the human system both at the population and clonal levels when T cells of infected individuals were activated with purified HA [22]. T cell clones isolated from influenza virus-infected individuals recognized both the 306–329 peptide and APC infected with the virus [23, 24]. The dominant epitope, recognized by T cell clones in the context of HLA-DR1 and of the related HLA-DR4 alleles [21, 22] was confined to the 306–318 sequence [25–27]. Analysis of X-ray crystallography data enabled the identification of contact residues of this peptide involved in the binding to HLA-DR1 [28]. Based on the recently described allele-specific motifs determined from the amino acid sequence of peptides eluted from MHC class II molecules [29], the 308–316 minimal core of the H3 sequence of influenza HA1 comprises a motif fitting to the grooves of the highly homologous human HLA-DR1 and murine I-E^d class II molecules, providing an explanation for their functional immunodominance in both species.

The peptide encompassing the 317–329 H1 HA1 sequence was also shown to induce a strong peptide-specific T cell response in BALB/c mice, although this region was identified as a subdominant I-E^d-restricted epitope in the secondary virus-specific response [10, 30, 31]. Extension of this peptide by the 1–13 N-terminal amino acids of the HA2 subunit (encompassing the fusion peptide, FP) gives rise to the intersubunit peptide, which resembles the 317–341 sequence of the immature form of HA0 (Table 1). This peptide, 25 amino acids in length, exhibits significantly increased immunogenicity and is able to elicit a protective immune response against the A/PR/8/34 influenza virus (H1N1) [30–33]. Peptide-specific T and B cells reacted not only with the corresponding peptide but recognized the whole A/PR/8/34 virus and other subtype variants and cross-reacted with peptides corresponding to other subtype sequences ([29, 30, 33] and Horváth et al., in preparation).

Here the following questions were addressed: (i) is local immunodominance of the 306–318 epitope over the 317–329 H3 region in the C-terminal HA1 stretch due to its more efficient binding to I-E^d molecules, (ii) are there subtype related differences in binding of the peptides covering distinct epitopes of the 306–329 region to MHC and/or in activating T cells, and (iii) what are the functional consequences of the close location of two epitopes competing for the same MHC class II peptide binding site. To explore these problems, *in vivo* immunization studies and *in vitro* assays performed with two T cell hybridomas as well as direct binding of N-terminal biotinylated peptides to MHC class II molecules expressed on living murine B cells were studied by flow cytometry [34–37]. Results of the binding assays were compared with those of the functional tests performed with polyclonal T cells elicited by the 306–329 peptides comprising both epitopes and with two T cell hybridomas of the Th1 phenotype characterized by fine specificity patterns identical to that of the 317–329-induced polyclonal T cell response [10, 30]. The binding data, which exhibited a good correlation with results of the functional assays, demonstrated that the 317–329 peptides comprising the H1 or H2 but not the H3 subtype sequences, are as efficient as the 306–318 peptides in binding to I-E^d molecules. These results show that local dominance of the 306–318 over the 317–329 epitope in the H3 sequence is explained by the less efficient binding of H3 317–329 to I-E^d as compared to the H1 and H2 peptides. In contrast to the H3 subtype, the corresponding epitopes comprising the H1 and H2 subtype sequences, have similar binding properties to I-E^d and T cell-activating capacity in context with the same MHC class II molecule. This observation may suggest that amino acid substitutions harbored in the H3 306–329 sequence can modify the relative binding capacity and invert the local hierarchy of immunodominance of two closely situated epitopes that are able to bind to the same restriction element. Based upon the strong similarity of the H-2 I-E^d and HLA-DR1 binding pockets and their corresponding peptide motifs [29, 38], the relevance of this finding to the human system is suggested.

2 Materials and methods

2.1 Synthetic peptides

The synthetic peptides listed in Table 1 were synthesized by the classical solid-phase tBoc method as described previously [30, 32, 33, 39, 40]. Peptides were purified by HPLC (peptide purity >96%) and characterized by amino acid analysis and mass spectroscopy. The 317–329 and 320–329 H3 peptides were synthesized with an automated multiple peptide synthesizer (Abimed model AMS U22, Langenfeld, Germany) using the company's protocols for N-α-fluorenylmethoxycarbonyl (Fmoc) synthesis. Following completion of chain assembly, the N-terminal Fmoc-protecting group was removed and N-terminal biotinylation was performed. Crude peptides were purified by reverse-phase HPLC on a semi-preparative C8 column (Lichrosorb RP-8, 7 μm, 250 × 10 mm, Merck, Darmstadt, Germany). Elution of peptides was achieved by linear gradients established between 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 75% acetonitrite in water (v/v). Purity of peptides was ascertained by analytical HPLC on RP-18 (Merck) and by amino acid analysis following exhaustive acid hydrolysis.

2.2 N-terminal biotinylation of peptides

The peptides listed in Table 1 were biotinylated as described before [35]. Brielfy, 2 mM peptide solution was prepared in 0.1 M $NaHCO_3$ and cooled in ice. 2-N-Hydroxysuccinimide-biotin (NHSB, 2 mg/ml; Sigma) dissolved in dimethylsulfoxide (DMSO) was added to 30% molar excess. The reaction was allowed to proceed at 0°C for 2 h. N-terminal biotinylation of the 317–329 and 320–329 H3 peptides with protected 326 Lys was performed in the resin bound form (Wang-resin, Nova biochem, Calbiochem AG, Laeufelingen, Switzerland). Peptide (11 mg) was suspended in a minimal volume of N-methyl-2-pyrollidone (NMP) and 15 μmol NHSB, and 1.5 μmol diisopropylethylamine were added. After 16 h, the reaction products were washed with NMP, methanol, and dry ether. The biotinylated peptide was deprotected and cleaved from the resin with a cleavage mixture containing 5% triethylsilane (Fluka Chemicals, Buchs, Switzerland), 5% water, and 90% TFA (v/v/v). After 2 h the cleaved peptide was precipitated with ice-cold, peroxide-free ether and following centrifugation the pellett was dissolved in water and subsequently lyophilized. The degree of biotinylation (as compared with non-biotinylated peptide) was estimated by HPLC fractionation on a R18 column using a spectroscopic assay based on the binding of 2(4-hydroxyazobenzene) benzoic acid to biotin [41]. The extent of biotinylation was >85%.

2.3 Cell lines and monoclonal antibodies

The murine B lymphoma lines A20 (ATCC TIB 208) and 2PK3 (ATCC TIB 203) were used as APC in the T cell activation and in the binding assays. The T cell hybridomas were developed from the spleen of BALB/c mice immunized either with the 317–341 (H1) peptide (H1-9-7-10) or with the 317–329 (H1) peptide and subsequently infected with the A/PR/8/34 influenza A virus (IP12-7 hybridoma) [30]. *In vitro* restimulation was performed with the corresponding peptides used for immunization and 5 days later activated T cells were fused with the BW-1100.129.237 $\alpha^-\beta^-$-thymoma cells [30]. The fusion partner was also used as a control in the cytofluorimetric assays. For II-2 quantitation, the IL-2-dependent CTLL-2 (ATCC TIB 214) or HT2 T cell lines were used.

The monoclonal antibody (mAb) 14-4-4S (mouse IgG2a; ATCC HB 32) specific for the I-E α-chain, was used in the binding or functional assays to verify MHC restriction. All cell lines were cultured in RPMI supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, antibiotics and 5% FCS (complete RPMI).

2.4 Peptide binding assay and cytofluorimetry

A20 or 2PK3 cells (5×10^5–1×10^6 cells suspended in 200 μl RPMI supplemented with 2 mM L-glutamine and 0.1% BSA or FCS) were incubated in standard FACScan tubes (Falcon) with 1–20 μM biotinylated peptides at 37°C for 4 or 14 h. A 4-h incubation is sufficient for peptide internalization to endosomal compartments and substitution of self peptides captured as instabile MHC class II-peptide complexes. A 14-h incubation, however, allows loading of newly synthesized MHC class II molecules [41–43]. Cells were washed twice with PBS containing 0.1% BSA and cooled in ice. Phycoerythrin-labeled streptavidin (Molecular Probes, OR) was added (2 μg/tube) and incubated for 30 min at 0°C. Cells were washed three times with cold PBS-0.1% BSA, resuspended in 0.5 ml washing solution and analyzed by flow cytometry using a FACScan analyzer (Becton Dickinson). Viable cells were gated on the basis of forward and side scatter or of exclusion of 7-amino-actinomycin D. Data are documented either as histograms or as increase in mean fluorescence (arbitrary units). In the latter case, control cells were incubated without biotinylated peptides under the same conditions as those incubated with the labeled ones and mean fluorescence of these samples were taken as background.

Inhibition of peptide binding by mAb, specific for the I-E^d α chain (14-4-4S), was performed by incubating the A20 or 2PK3 cells with biotinylated peptides in the presence of mAb-containing supernatant. In the peptide competition assays, cells were pre-incubated with a 10–100 molar excess of unlabeled competitor peptides for 4 h at 37°C as described above. Bitionylated peptides were added at limited concentration given in the figure legends and the samples were further incubated for another 10 h. Labeling was performed as described for the direct binding assays.

2.5 Monitoring T cell activation by IL-2 production

T hybridoma cells (2×10^4 cells) were cultured in 96-well flat-bottom tissue culture plates (Nunc) in complete RPMI in the presence of different concentrations of peptides and 5×10^4 A20 or 2PK3 APC. In the competition assay with mAb, different dilutions of mAb-containing cell culture supernatants were added together with the peptides. Subsequently 75 μl of culture supernatants were removed at 24 h of culture and transferred to secondary cultures where the amount of secreted IL-2 was measured by the proliferation of CTLL-2 or HT2 detector cells. In this assay, the indicator were used at 4×10^3 (CTLL) or 4×10^4 (HT2) cells/well starting density and cell proliferation was measured by addition of [^{3}H] thymidine or 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), respectively [30].

2.6 Polyclonal T cell activation

Spleen and lymph node cells of BALB/c mice immunized with 100 μg peptide were isolated on days 18–21 and the T cell activating capacity of the peptides *in vitro* was measured by the standard proliferation assay as described previously [30]. The titer of peptide-specific Ab was measured by solid-phase indirect EIA as described [30, 31].

Table 1. The location, code, amino acid sequence and activity of the synthetic peptides comprising the 306–341 intersubunit region of immature HA0 to activate the I-E^d restricted H-1-9-7-10 and IP12-7 T cell hybridomas[a)]

Location	Code subtype	Sequence	Activation H1-9-7-10	IP-12-7
$HA1_{306-318}$	306–318 (H1)	**PKYVRSAKLRMVT**	–	–
$HA1_{306-318}$	306–318 (H2)	**PKYVKSEKLVLAT**	–	–
$HA1_{306-318}$	306–318 (H3)	**PKYVKQNTLKLAT**	–	–
$HA1_{317-329}$+$HA2_{1-12}$	317–341 (H1)	**VTGLRNIPSIQSRGLFGAIAGFIEG**	+++	+++
$HA1_{317-329}$+$HA2_{1-12}$	317–341 (H2)	**ATGLRNVPQIESR GLFGAIAGFIEG**	+++	–
$HA1_{317-329}$+$HA2_{1-12}$	317–341 (H3)	**ATGMRNVPEKQTRGLFGAIAGFIEG**	–	–
$HA1_{306-329}$+$HA2_{1-12}$	306–341 (H1)	**PKYVRSAKLRMVTVTGLRNIPSIQSRGLFGAIAGFIEG**	+++	+++
$HA1_{306-329}$+$HA2_{1-12}$	306–341 (H2)	**PKYVKSEKLVLATATGLRNVPQIESRGLFGAIAGFIEG**	+++	–
$HA1_{306-329}$+$HA2_{1-12}$	306–341 (H3)	**PKYVKQNTLKLATATGMRNVPEKQTRGLFGAIAGFIEG**	–	+
$HA1_{306-329}$	306–329 (H1)	**PKYVRSAKLRMVTVTGLRNIPSIQSR**	+++	+++
$HA1_{306-329}$	306–329 (H2)	**PKYVKSEKLVLATATGLRNVPQIESR**	+++	–
$HA1_{306-329}$	306–329 (H3)	**PKYVKQNTLKLATATGMRNVPEKQTR**	–	N.T.
$HA1_{317-329}$	306–329 (H1)	**VTGLRNIPSIQSR**	+++	+++
$HA1_{317-329}$	317–329 (H2)	**ATGLRNVPQIESR**	+++	–
$HA1_{317-329}$	317–329 (H3)	**ATGMRNVPEKQTR**	–	+
$HA1_{318-327}$	318–327 (H1)	**VTGLRNIPSI**	–	+
$HA1_{319-328}$	319–328 (H1)	**GLRNIPSIQS**	–	–
$HA1_{319-328}$	319–328 (H2)	**GLRNVPQIES**	–	–
$HA1_{319-328}$	319–328 (H3)	**GMRNVPEKQT**	–	–
$HA_{320-328}$	320–328 (H1)	**LRNIPSIQS**	–	–
$HA_{320-328}$	320–328 (H2)	**LRNVPQIES**	–	–
$HA_{320-328}$	320–328 (H3)	**MRNVPEKQT**	–	–
$HA_{320-329}$	320–329 (H1)	**LRNIPSIQSR**	–	–
$HA_{320-329}$	320–329 (H2)	**LRNVPQIESR**	–	–
$HA_{320-329}$	320–329 (H3)	**MRNVPEKQTR**	–	–
$HA2_{1-12}$R	FP3	**GLFGAIAGFIEGR**	–	–

a) Activation of the T cell hybridomas was measured by titration of IL-2 in cell culture supernatants after culturing the cells in the presence of 2PK3 APC at different concentration of the peptides. Relative efficiency of the peptides was characterized by their concentrations required for induction of 50% maximal IL-2 production that was determined from the dose-response curves: 0.1 μM (+++), 1 μM (++) and 50 μM (+).

3 Results

3.1 Biological activity of synthetic peptides covering the 306–341 region of influenza virus HA

Table 1 summarizes the functional activity of the H1-9-7-10 and IP12-7 T cell hybridomas in the presence of unlabeled peptides and of the 2PK3 murine B lymphoma cells expressing I-E^d molecules. Although both T cell hybridomas were elicited by immunization with peptides encompassing the 317–329 H1 sequence, they show a distinct cross-reactivity pattern with the corresponding H2 and H3 peptides. The IP12-7 T cell hybridoma can be activated by peptides comprising the 317–329 H1 sequence. It does not react with the corresponding H2 peptide but shows slight cross-reactivity with the 317–329 H3 peptide (Table 1). The H1-9-7-10 T cell hybridoma recognizes peptides of different length covering both the H1 and H2 317–329 sequences but does not recognize any of the H3 peptides (Table 1).

N-terminal truncation of the 317–329 peptides abolished their T cell-activating capacity but elimination of the C-terminal Arg did not (Table 1). Both hybridomas recognize and can efficiently be activated with peptides extended with the 306–318 sequences, *i.e.* 306–329 and 306–341 (Table 1). These functional properties provided a good tool for analyzing the relative activating and inhibitory activity of peptides covering various regions of the 306–329 subtype sequences.

Synthetic peptides covering the 306–341 sequence of the H1, H2 and H3 HA sequences were biotinylated and their biological activity was tested in comparison to the parental peptides by IL-2 production of the T cell hybridomas (data not shown). No alteration in the functional activity of peptides was observed as a result of N-terminal biotinylation. Therefore, these modified peptides were useful reagents for studying their direct MHC binding.

3.2 Direct binding of peptides to MHC class II molecules of living A20 and 2PK3 cells

Fig. 1 presents results of flow cytometric measurements of peptide binding performed after 14-h incubation at 37 °C. The figure shows the increase in mean fluorescence values (arbitrary units) of A20 or 2PK3 cells incubatd with 20 μM biotinylated 317–329 peptide (H1 subtype) (Fig. 1B) in comparison to cells labeled with the I-Eα-specific monoclonal antibody 14-4-4S (Fig. 1A). Results summarized in Fig. 1C, D show the specificity of peptide binding to A20 or 2PK3 cells. Binding of biotinylated 317–329 peptide to A20 (Fig. 1CIII) is completely inhibited by the mAb 14-4-4S (Fig. 1CI) as compared to the corresponding negative control (Fig. 1CII). Partial inhibition of biotinylated

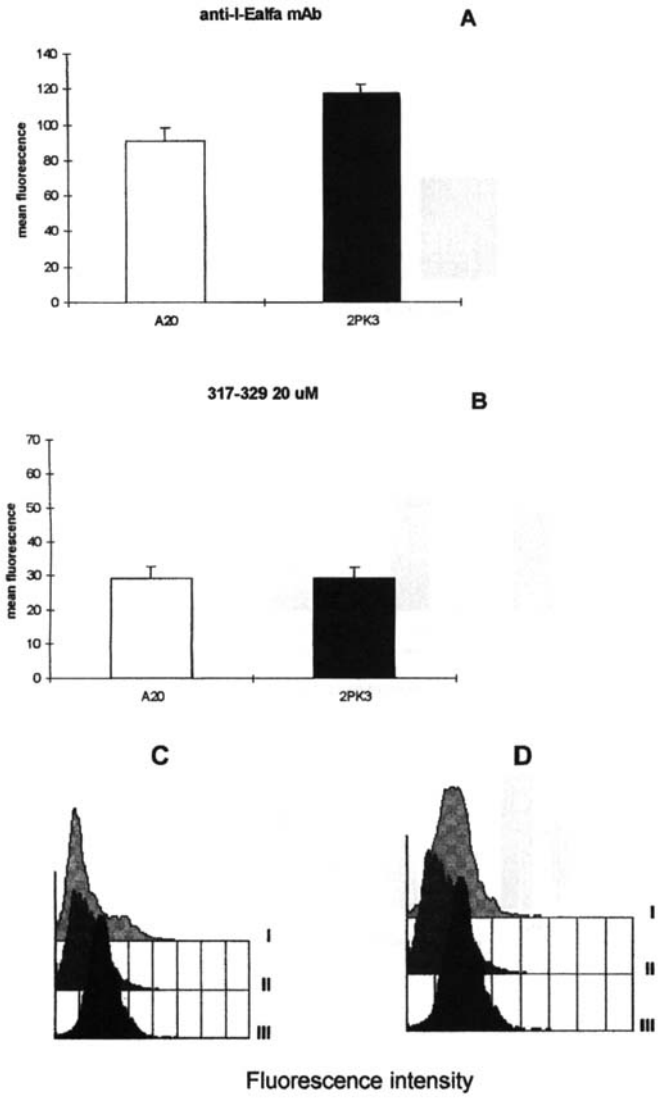

Figure 1. Flow cytometric binding measurements of MHC class II-specific mAb (A) or biotinylated HA peptides (B–D) to A20 and 2PK3 cells. The increase of mean fluorescence was measured after incubation with 1 μg biotinylated 14.4.4 mAb for 1 h on ice (A), with 20 μM N-terminal biotinylated 317–329 (H1 subtype) (B) for 14 h at 37 °C, followed by 30-min incubation with PE-labeled streptavidin on ice. The increase of mean fluorescence was calculated as a difference of antibody treated or peptide loaded samples and negative controls incubated without the antibody or peptides. Mean values of three to five independent experiments ± SD are given. Binding specificity was tested by Ab (C) and peptide (D) competition with A20 or 2PK3 cells, respectively. Binding of 317–329 (H1 subtype) peptide (20 μM) to A20 (C/III) or 2PK3 (D/III) cells is presented with the corresponding negative controls (C/II, D/II) and with cells incubated with the same peptides in the presence of 14.4.4 mAb (C/I) or 20-fold molar excess of 320–328 (H1) peptide (D/I).

317–329 peptide binding (Fig. 1DIII) to 2PK3 cells was found in the presence of a 20-fold molar excess of unbiotinylated 320–328 peptide (Fig. 1DI) as compared to the negative control (Fig. 1DII). As neither the biotinylated 14-4-4S mAb nor the biotinylated peptides bind to BW5147.3 cells (data not shown), these results underscore the binding specificity of N-terminal biotinylated peptides to I-E^d molecules on the surface of living A20 or 2PK3 cells.

3.3 Comparative binding efficiency of peptides comprising various regions of the 306–341 HA subtype sequences

Peptides comprising the three subtype sequences of the 306–318, 317–329 and 317–341 regions bind to 2PK3 cells with slightly different efficiency (Fig. 2). The inability of the H3 IP peptide (IP3) to bind to 2PK3 or A20 cells, however, could be the consequence of biotinylation and functional alteration of the Lys residue at position 326 which is unique in the H3 sequence (Table 1). This possibility was further investigated using shorter peptide analogs of IP. As summarized in Fig. 2C all analogs encompassing the H3 sequence have a detectable but lower binding capacity compared to that of the H2 or H1 sequences. To rule out the involvement of Lys326 moification in binding to I-E^d, binding measurements of the 317–329 and 320–329 peptides, having protected Lys residues during the biotinylation procedure, were also performed. These peptides had similar, low binding activity to living cells as the ones labeled by the standard method (data not shown), suggesting that their lower binding activity is rather due to differences in peptide sequences. This interpretation was further confirmed by the observed similar activity of the unlabeled and biotinylated 317–329 H3 peptide in an activation assay performed with IP 12-7 T cells in the presence of 2PK3 APC (data not shown).

In good correlation with the binding data summarized in Fig. 2 no activation of the H1-9-7-10 hybridoma could be achieved by any of the H3 peptides and the dose-response curve of the 317–329 H1 and H3 peptides revealed that a 100-fold higher concentration of the H3 peptide was required to reach maximal IL-2 production of the IP12-7 hybridoma (data not shown). The reduced ability of the 317–329 H3 peptide compared to its H1 analog to induce IL-2 production can be explained by its lower binding to I-E^d molecules. Still the role of the T cell receptor should also be considered. The results showing that the 317–329 H2 peptide, despite of its efficient binding to MHC class II molecules, does not activate the IP12-7 T cell hybridoma (Table 1) but has comparable activity to the H1 peptide with the H1-9-7-10 hybridoma, clearly point to to the role of subtype-related amino acid substitutions in contact both with the MHC and the T cell receptor.

Data summarized in Fig. 2C show that truncations at both termini of the parental 317–329 peptides result in analogs which still bind to I-E^d despite of their inactivity in the functional assay (Table 1). Furthermore, comparing the binding activity of peptides with or without C-terminal Arg revealed that this additional amino acid increases the efficiency of MHC binding (Fig. 2C). The 320–329 peptides which retained their MHC binding capacity but lost functional activity were used in the functional and binding inhibition assays to assess relative binding to MHC class II molecules.

3.4 Competition between the 306–318 and 319–329 peptides for binding to I-E^d

The relative binding capacity of the 306–318 and 319–329 H1 peptides was studied in both binding and functional competition assays. As is summarized in Fig. 3A, a similar

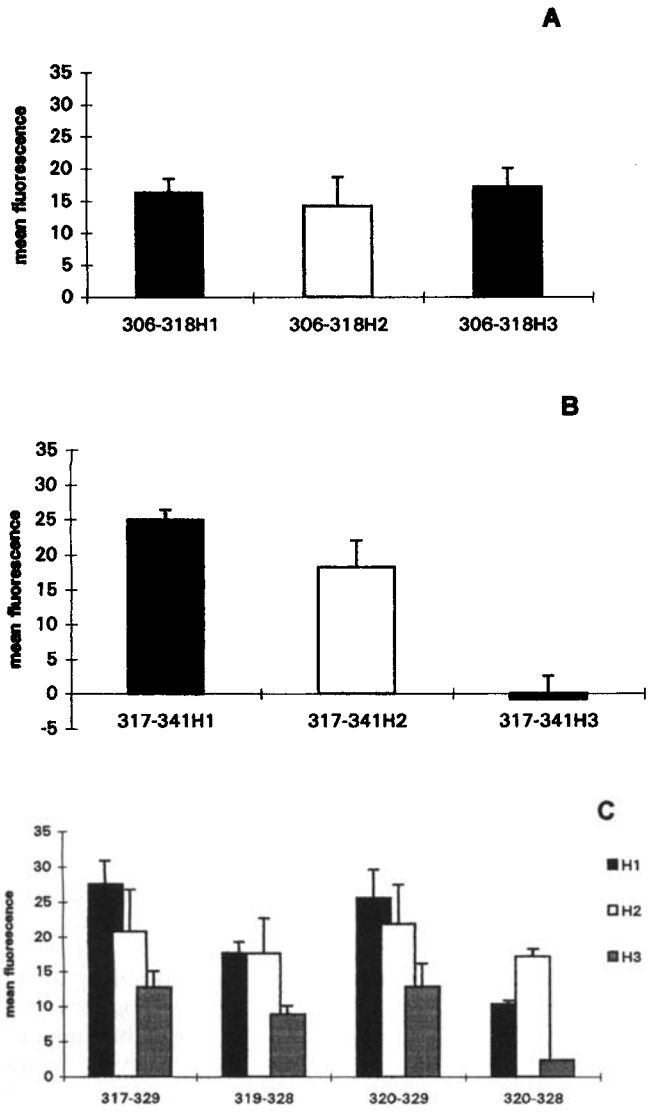

Figure 2. Flow cytometric assay of the binding of peptides comprising subtype sequences of the 306–341 region of HA to I-E^d molecules. The shift in mean fluorescence (arbitrary units) as a result of 14-h incubation at 37 °C of 2PK3 cells with 20 μM N-terminal biotinylated peptides was measured in three independent experiments and mean values ± SD are shown.

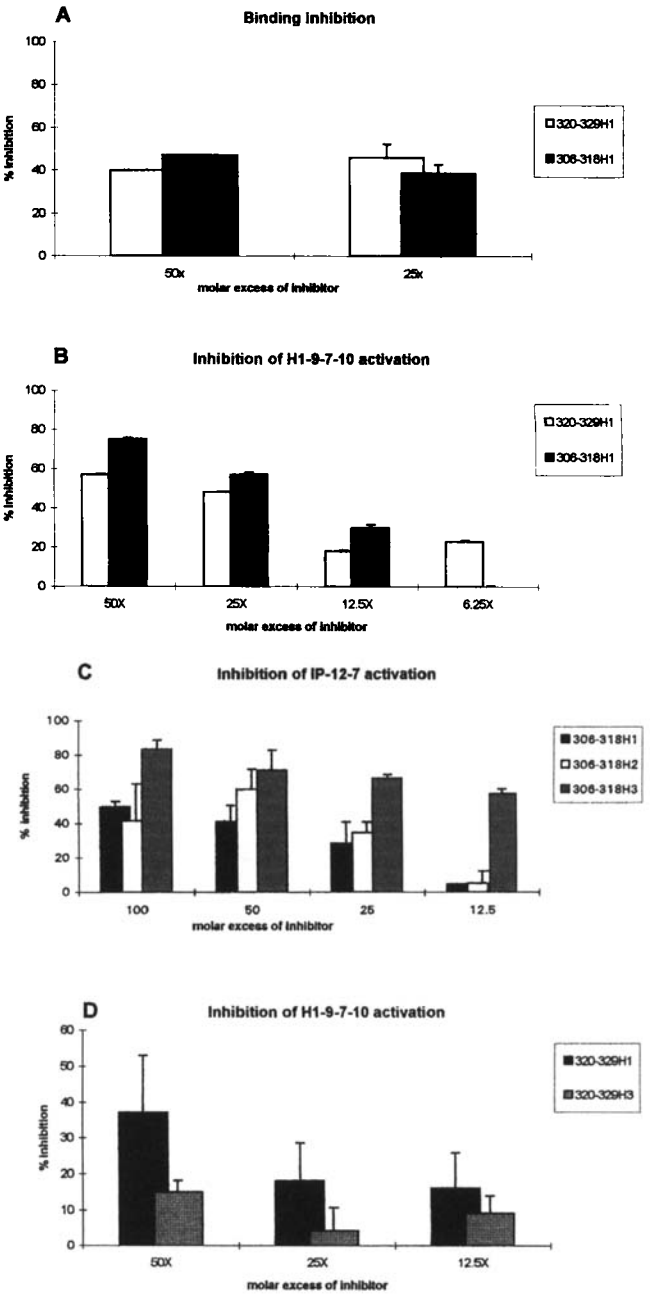

Figure 3. Inhibition of peptide binding to MHC (A) and IL-2 production of T cell hybridomas (B–D) by HA peptides comprising two different epitopes. (A) A20 cells (5×10^5) were preincubated with 25- or 50-fold molar excess of competitor peptides and the binding of 4 μM biotinylated peptide was detected; (□) 320–329 H1, (■) 306–318 H1. Values shown represent the mean fluorescence intensity (mean ± SD) of three independent experiments and are given as percent inhibition. (B) H1-9-7-10 T cells (10^4–2×10^4) were incubated with 2×10^4 2PK3 cells as APC at a suboptimal concentration (0.5–1 μg/ml) of 317–319 H2 peptide in the presence of increasing concentrations (6- to 100-fold molar excess) of the non-activating 320–329 H1 (□) or 306–318 H1 (■) peptides. (C) As for (B) using 104–2×10^4 IP 12-7 T cells and 2×10^4 A20 cells as APC, with 317–329 H1 peptide and increasing concentrations of 306–318 H1 (■), 306–318 H2 (□), and 306–318 H3 (■). (D) As for (B) using H1-9-7-10 T cells with 2PK3 as APC, in the presence of 317–329 H2 peptide and increasing concentrations of 320–329 H1 (■) and 320–329 H3 (■) peptides. T cell activation was detected by measuring IL-2 production using CTLL-2 or HT2 indicator cells.

degree of inhibition could be achieved if the binding of 20 μM biotinylated 306–318 or 317–329 peptide was measured in the presence of unlabeled 320–329 and 306–318 peptides, respectively. Competition of non-activating peptides for I-E^d binding sites was also demonstrated in functional assays as demonstrated in Fig. 3B–D. The H1-9-7-10 (Fig. 3B, D) T cell hybridoma was activated in the presence 2PK3 cells with a limited concentration of 317–329 H2 activator peptides together with different concentrations of non-activating 306–318 and 320–329 competitor H1 peptides (Fig. 3B). Similar to the binding inhibition assay, the two peptides possessed similar inhibitory capacity in this assay. The molar excess required for 50 % inhibition in both the binding and functional inhibition assays was in the same order of magnitude (Fig. 3A, B).

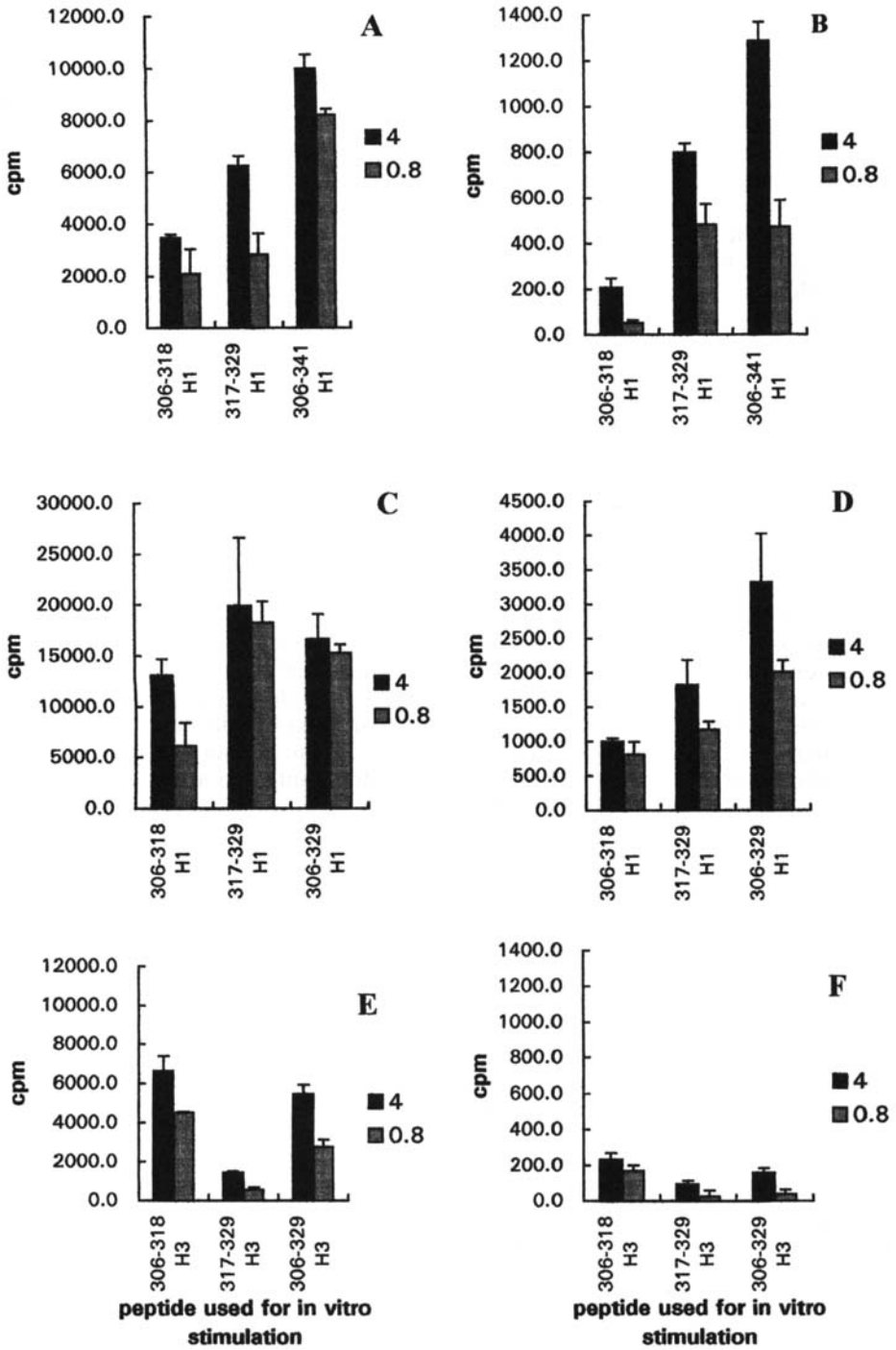

Figure 4. Specificity of *in vivo* activated polyclonal T cells induced by peptides comprising both epitopes. Spleen (A, C, E) and lymph node (B, D, F) cells of BALB/c mice immunized with 100 μg 306–341 (H1 (A, B), 306–329 H1 (C, D) or 306–329 H3 (E, F) peptides were activated *in vitro* by 4 μg/ml (dark columns) or 0.8 μg/ml (gray columns) peptides in the presence of irradiated syngeneic spleen cells. Proliferation was measured by [^{3}H] thymidine incorporation.

Fig. 3C, D demonstrates the relative inhibitory capacity of the 306–318 and 320–329 peptides tested in the sensitive inhibition assay performed with the IP12-7 (Fig. 3C) or the H1-7-9-10 (Fig. 3D) hybridomas. Fig. 3C demonstrates the highest efficiency of the 306–318 H3 peptide in contrast to the low inhibitory activity of the 320–329 H3 peptide (Fig. 3D) in the functional inhibition assay.

3.5 T cell-activating capacity *in vivo* of the 306–318 and 317–329 epitopes

Spleen (Fig. 4A, C, E) and lymph node (Fig. 4B, D, F) cells of peptide immunized BALB/c mice were separated, depleted for B cells and proliferation as well as IL-2 production of *in vitro* re-stimulated T cells was measured. T cells of mice immunized either with the 306–341 (Fig. 4A,

B or 306–329 (Fig. 4C, D) H1 peptides responded to both the 306–318 and the 317–329 peptides and the most intense response was detected with the peptide encompassing both epitopes (Fig. 4A–D). Similar results were obtained with the H2 peptides (data not shown). However, immunization with the 306–329 H3 peptide (Fig. 4E, F) resulted in T cell responses of similar intensity to the 306–329 and to the 306–318 peptides, but no reactivity with the 317–329 peptide was observed. These results demonstrate that under *in vivo* conditions both epitopes, located in the 306–318 and the 317–329 H1 regions, are able to activate peptide-specific T cells. In contrast to H1, the 306–329 H3 sequence has only one functional T cell epitope comprised in 306–318 (Fig. 4E, F).

4 Discussion

Immunodominance of T cell epitopes may have an essential role in the development of T cell responses to pathogens and vaccines. Peptide affinity to MHC has been shown as a major factor in immunogenicity which limits the number of specific peptide/MHC complexes presented to T cells and determines competition among peptides for binding to MHC molecules [1, 44]. However, binding of peptides to MHC with low affinity may have an important role in tolerance induction and shaping immunodominance [45]. The hierarchy of immunodominance in large proteins, however, is a complex interplay of different factors including efficiency and selectivity of antigen processing and presentation, the size of the peripheral T cell repertoire and the local concentration of cytokines required for T cell expansion and differentiation [2].

The influenza HA subunits are generated by a posttranslational modification mediated by host-cell derived subtilisin-like proteases [46, 47]. The C-terminal stretch of HA1, although being embedded in the core of the protein, encompasses an enzyme susceptibility motif which is the target of a special subtilisin-like enzyme [46, 47]. This enzymatic cleavage, which plays a pivotal role in the infectivity of the virus, initiates, in collaboration with the pH-induced conformational change of HA, the further degradation of the C-terminal segment. The C-terminal region of HA1 has been described as a dominant epitope which involves multiple T cell epitopes recognized both in the human and mouse systems [9, 10, 14, 17, 18, 22–24]. The immune response elicited by a conformationally stabilized peptide 317–341, which comprises the HA1 317–329 H1 sequence, confers protection against lethal infection with the A/PR/8/34 virus ([10, 30–32] and Horváth et al., in preparation). The subtype-specific but conserved amino acid sequence and the functional importance of this region in enzymatic cleavage prompted us to study the binding capacity of peptides comprising distinct but not overlapping epitopes of this region to the same murine MHC class II molecule and compare their ability to induce T cell activation *in vitro* and *in vivo*. This study was extended by monitoring the effect of natural amino acid substitutions, evolved during the phylogeny of virus subtypes, on these properties. *In vitro* experiments measuring peptide binding to I-E^d molecules expressed on murine B lymphoma cells and functional assays carried out with two murine T cell hybridomas recognizing the 317–329 region with distinct fine specificity ([30] and this study) as well as *in vivo* immunization studies with peptides comprising both the 306–318 and 317–329 sequences were performed to assess the local immunodominance of these adjacent epitopes.

Our results can be summarized as follows: (i) flow cytometric monitoring of cell surface bound N-terminal biotinylated peptides in combination with binding and functional inhibition assays provides semiquantitative data on their binding capacity, (ii) based on the binding and functional data, the 306–318 and the 317–329 peptides encompassing the H1, H2 but not the H3 sequences bind with an apparent similar affinity to, and therefore, compete for the same restriction element, *i.e.* I-E^d, (iii) the 317–341, the 317–329 peptides and their truncated analogs show subtype-dependent differences in I-E^d binding and (iv) differences in the binding capacity of peptides comprising the three subtype sequences provide a rationale for the local immunodominance of the 306–318 region over the 317–329 epitope upon peptide immunization in the case of the H3 but not in the H1 or H2 sequences.

According to previous experiments [36, 37], all documented binding assays were performed after incubation of living APC with the labeled peptides for 14 h at 37 °C. The long-incubation protocol was designed to detect stable MHC class II-peptide complexes (half-life 7–12 h) generated as the net result of different peptide loading pathways [42, 43]. These include direct interaction of peptides with short-lived instable complexes [47–49] as well as the HLA-DM- and invariant chain-dependent peptide binding occurring in endosomal compartments [50, 51].

The local immunodominance of the 306–318 and 317–329 epitopes is determined by the subtype-specific sequence of this region as revealed by the relative binding efficiency, competitor activity and immunogenicity of the corresponding peptides comprising the H1, H2 or H3 subtype sequences. The H1, H2 and H3 306–318 peptides bind to I-E^d with an apperantly similar affinity (Fig. 2) and elicit proliferating T cells *in vivo* in case of all subtype sequences (Fig. 4). The H1 and H2 sequences of 317–329 residues were also shown as potent I-E^d binding epitopes (Fig. 2, Table 1). The 317–329 and 317–341 peptides, however, show subtype related differences in their MHC binding (Fig. 2) and in their efficiency to activate (Table 1), inhibit (Fig. 3A–D) or induce *in vivo* T cell responses (Fig. 4). The discrepancy between the lack and the relatively low, but detectable binding and activating capacity of the 317–341 and 317–329 H3 peptides, respectively (Fig. 2) may be the consequence of the strikingly different solution conformation of the fusion peptide extended analogs [33, 39, 40]. The high propensity of 317–341 to adopt an ordered conformation in these peptides is mediated by FP but may affect the orientation of 317–329 side chains influencing both the occupancy of the corresponding pockets and/or the contact residues with the TCR. This explanation is supported by the finding that the elimination of the fusion peptide from the C-terminal of 317–341 resulted in a tridecapeptide 317–329, which showed some binding to I-E^d (Fig. 2). In addition, both the 306–318 and 317–329 H1 or H2 peptides were recognized by monoclonal (Table 1) or polyclonal T cells (Fig. 4) in longer peptides extended by N- or C-terminal natural sequences, providing evidence that the flanking sequences have no

inhibitory effect on the T cell recognition of the 317–329 epitope. Control experiments ruled out the possibility that the low binding of 317–329 H3 was merely due to the biotinylation of the unique Lys at position 326.

These results demonstrate the similar activity of the 306–318 and 317–329 H1 or H2 peptides and the poor activity of the 317–329 H3 peptide in the binding (Figs. 1, 2), activation (Table 1) or inhibition assays (Fig. 3) and in the immunization experiments (Fig. 4). The sensitive functional inhibition assays demonstrated an inverse rank order of peptides in their relative competitor efficiency of the 306–318 and 317–329 subtype peptides, respectively (Fig. 3D, E). Our results confirm previous data that relative binding efficiency to MHC molecules is a critical factor in determinant selection and ranking peptide immunogenicity [1, 42, 43, 52]. The subdominant feature of the 317–329 peptide upon viral infection may be the consequence of high antigenic competition, inappropriate antigen processing, or the low frequency of T cells of this specificity. This hierarchy, however, can be altered by peptide pre-immunization which may increase the frequency of T cells of the desired specificity. Using the 306–329 or 306–341 peptides for pre-immunization, the ratio of T cells against the two adjacent epitopes was determined by their relative binding efficiencies (Fig. 4).

Extension of the 317–329 peptide by the hydrophobic fusion peptide (306–341 or 317–341) significantly increases the immunogenicity of the subdominant 317–329 epitope ([30–32] and Horváth et al., in preparation) and preimmunization with this peptides results not only in the activation of Th1 cells but also in antibody production and elevated protection against lethal A/PR/8/4 virus infection ([31, 32] and Horváth et al., in preparation). Th1 cells elicited by peptide immunization not only produce IL-2 but also secrete large amounts of TNF upon activation by peptide or virus-pulsed APC (Horváth et al., unpublished results). TNF in combination with IFN-γ was demonstrated to confer strong antiviral activity [53, 54] and mediate apoptosis, and may, therefore, be involved in the elimination of virus-infected cells.

Our results confirm previous data which demonstrated that immunization with the 305–328 H3 peptide resulted in a T cell response mediated primarily by the 305–318 epitope and by an antibody response directed against regions 314–318 and 320–328 [17, 18, 55]. In contrast to H3, immunization with the 306–329 or 317–341 H1 peptides elicited both 306–318- and 317–329-specific T cells and antibodies against the 317–329 region [30, 31]. The importance of this local epitope hierarchy in response to the whole virus may depend on multiple factors. Nevertheless, enhancement of the immune response by peptide preimmunization to a subdominant protective epitope may be a beneficial component of the antiviral immune response and preimmunization with the 306–341 or 317–341 synthetic peptides comprising H1 or H2 sequences confers protection against lethal influenza virus infection ([31, 32] and Horváth et al., in preparation).

This work was supported by the grants OTKA no. 017157, AKP no. 33115/6 and OMFB no. E0028/960202 to É.R and grant B104-CT96-0135 from the European Community to I.P. The expert technical assistance of Erzsébet Veress is acknowledged.

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