Characterization of a new H-2D<sup>k</sup>-restricted epitope prominent in primary influenza A virus infection

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**Introduction**

Infection with influenza A viruses elicits a vigorous CD8<sup>+</sup> T cell response in mice (Flynn et al., 1998). Because of the convenience of this experimental model and the early availability of cloned influenza virus gene segments, much of our current knowledge about antigen presentation to CD8<sup>+</sup> T lymphocytes has come from studies using influenza virus (Parker & Gould, 1996). In particular, influenza virus infection of mice has been useful in investigating the causes of immunodominance, a central feature of CD8<sup>+</sup> T cell responses that enable the rescue of defined influenza virus mutants from a recombinant DNA system (Fodor et al., 1999; Neumann et al., 1999) should ensure that the virus continues to be an important experimental model for studying the mechanisms of antigen presentation.

Influenza virus epitopes that are recognized by CD8<sup>+</sup> T lymphocytes in H-2<sup>b</sup> and H-2<sup>d</sup> mice have been well-characterized and their relative immunodominance has been investigated thoroughly (Vitiello et al., 1996; Belz et al., 2000; Chen et al., 2000). However, much less is known about the CD8<sup>+</sup> T cell response to influenza virus in H-2<sup>k</sup> mice. Previously, we have investigated the specificity of the CTL response to influenza virus A/PR/8/34 in CBA/Ca (H-2<sup>k</sup>) mice and defined four different epitopes presented by the K<sup>k</sup> molecule: two epitopes in the haemagglutinin (HA), one epitope in the nucleoprotein (NP) and one epitope in the non-structural protein NS1 (Gould et al., 1991; Cossins et al., 1993). Although it has long been thought that epitopes in the NP are immunodominant, the dominance hierarchy of the K<sup>k</sup>-restricted epitopes has not been determined, and there is also an additional, uncharacterized D<sup>k</sup>-restricted response to influenza virus in H-2<sup>k</sup> mice. This response was described as long ago as 1979 (Blanden et al., 1979), but as yet no influenza virus peptide epitopes presented by the D<sup>k</sup> major histocompatibility complex (MHC) class I molecule have been reported. Other studies have confirmed a prominent D<sup>k</sup>-restricted response to influenza virus infection (Stringfellow et al., 1983) and to the PB1 polymerase protein in H-2<sup>k</sup> mice (Bastin et al., 1987; Bennink et al., 1987; Bennink & Yewdell, 1988; Reay et al., 1989) and demonstrated, using T cell hybridomas, that there is a D<sup>k</sup>-restricted response to PB1 (Daly et al., 1995).

In this study, we have identified a D<sup>k</sup>-restricted epitope derived from PB1 and shown, in polyclonal cell lines, that a large majority of CD8<sup>+</sup> T cells recognize this peptide, implying that this is the major epitope within the influenza virus PB1...
protein. Enzyme-linked immunospot (ELISpot) experiments demonstrated that this new epitope is at least as immunodominant during primary influenza virus infection of H-2k mice as any of the previously defined Kk-restricted epitopes.

**Methods**

- **Generation of PB1-specific CD8+ T cell lines.** Female 8- to 12-week-old CBA/Ca mice (Harlan) were immunized with 10⁵ p.f.u. of recombinant vaccinia virus (VV) PB1-VAC (Smith et al., 1987) by intravenous injection. Spleens were removed 2 weeks post-infection and spleen cells were then restimulated in vitro using influenza virus A/PR/8/34-infected feeder spleen cells, as described previously (Towndsend et al., 1984; Gould et al., 1991). Effector cells were subsequently restimulated with antigen at weekly intervals in the same way and after 3 weeks of culture in vitro, human recombinant interleukin-2 (Cetus Corp) was added to give a final concentration of 10 U/ml. Effector cells were used in assays on day 4 or 5 after restimulation with antigen.

- **Recombinant VV.** PB1-VAC, a recombinant WR strain expressing the PB1 polymerase of influenza virus A/PR/8/34 has been described previously (Smith et al., 1987). Four new recombinant VV expressing overlapping fragments of PB1 were constructed. PB1 fragments were inserted into the thymidine kinase (tk) gene of VV using the shuttle vector pKG18 (Gould et al., 1991). PB1 DNA fragments were generated by PCR using Phi polymerase (Stratagene), PB1-specific oligonucleotides containing restriction enzyme recognition sites and the plasmid that was used to make PB1-VAC as template DNA. The new recombinant viruses were designed to express influenza virus A/PR/8/34 PB1 gene fragments comprising amino acid residues 1–150, 1–300, 1–450 and 1–600. All plasmid constructs were confirmed by DNA sequencing using a Perkin Elmer ABI 373 DNA sequencer. Recombinant viruses were also assessed for the presence of the VV tk gene by PCR using viral genomic DNA and oligonucleotide primers specific for the tk gene (5′ AATAGACGAGTTAGACG 3′ and 5′ ATTCGTTTCTCACCACCC 3′).

- **Synthetic peptides.** An overlapping set of 71 different peptides, each nine amino acid (9-mer) residues in length, was synthesized by Chiron Mimotopes. Individual peptides were supplied by Research Genetics. Peptides were dissolved in RPMI 1640 medium for use in assays.

- **Cytotoxic assay.** A standard 5 h ³⁵Cr-release assay was used with modifications for adherent target cell lines, as described previously (Towndsend et al., 1984; Gould et al., 1991). The target cell lines L929 (Kk), 3T3 (Kk), C3H-10T1/2 (Kk) and 32D (Kk) (Bennink & Yewdell, 1988) were used. Virus infections of target cells, use of synthetic peptides and calculation of percentage of specific lysis were all as described previously (Gould et al., 1991). The average values of all experimental points, which were in duplicate with quadruplicate controls, are shown.

- **Intracellular interferon (IFN)-γ staining.** Effector T cell lines were incubated for 5 h at 37 °C either without peptide or with 10 μg/ml of peptide ARLGKYGMF in DMEM containing 10% foetal bovine serum (FBS) and 2 μg/ml of brefeldin A (Sigma). Cells were then washed and stained with anti-CD8α-cyochrome (Pharmingen) for 30 min on ice. After incubation, cells were washed in PBS containing 3% FBS and 0.1% NaN₃ and fixed with 4% formaldehyde in PBS for 20 min. After washing, cells were permeabilized with 0.5% saponin (Sigma) in PBS for 10 min, centrifuged and resuspended in 50 μl of 0.5% saponin in PBS. Cells were then stained with anti-IFN-γ-PE (Pharmingen) for 20 min. After incubation, cells were washed in PBS containing 3% FBS and 0.1% NaN₃ and analysed on an EPICS XL flow cytometer using Expo 2 software (Beckman Coulter).

- **ELISpot assay.** Female 8- to 10-week-old CBA/Ca mice were infected with 0.05 HAU influenza virus A/PR/8/34 in 50 μl PBS by intranasal inoculation or with 300 HAU in 500 μl PBS by intraperitoneal injection. After 8 days (Belz et al., 2000), the numbers of IFN-γ-producing cells in spleen cell populations from individual mice were determined by ELISpot analysis (Power et al., 1999). Nitrocellulose-bottomed 96-well plates (Millipore) were coated for 2 h at 37 °C followed by overnight incubation at 4 °C with rat anti-mouse IFN-γ antibody (clone R4-6A2; Pharmingen). Dilutions of responder spleen cells in complete medium were cultured without or with 10 μM peptide epitope for 48 h. Plates were then washed and incubated with biotinylated IFN-γ antibody (clone XMG1.2; Pharmingen) followed by streptavidin conjugated to alkaline phosphatase (Boehringer Mannheim). Spots were visualized using BCIP/NBT alkaline phosphatase substrate (Promega) and counted using an automated ELISpot plate counter (Autoimmun Diagnostika). Test wells were assayed in triplicate and the frequency of peptide-specific T cells present was calculated by subtracting the mean number of spots obtained in the presence of no peptide from the mean number of spots obtained in the presence of peptide.

IFN-γ ELISpot assay was also used to confirm the MHC restriction of the PB1 peptide epitope by using restimulated PB1-specific effector CTL (as responder cells), peptide epitope and transfected P815 feeder cells (H-2b haplotype) expressing either Dk or Kk MHC class I molecules. P815-Dk cells were obtained from Diane Scott, Imperial College School of Medicine, London, UK. Stable transfectant P815-Kk cells were generated by electroporation of P815 cells with an expression plasmid containing a full-length Kk cDNA under the control of the SV40 early promoter. MHC class I expression in both cell lines was verified by flow cytometry using specific antibodies.

**Results**

- **The CD8+ T cell response to PB1 is Dk-restricted**

As a result of our previous work on Kk-restricted epitopes in influenza virus (Gould et al., 1991; Cossins et al., 1993) and the reported existence of a Kk-restricted response to the PB1 polymerase protein (Bennink & Yewdell, 1988), we attempted initially to identify a Kk-restricted epitope in the influenza virus A/PR/8/34 PB1 protein. A total of 20 different PB1 peptides containing the consensus motif for Kk-presented peptides (Gould et al., 1991; Norda et al., 1993; Brown et al., 1994) was made, but none of these peptides was recognized by our H-2k, PB1-specific T cell lines (data not shown). These PB1-specific T cell lines were derived by priming CBA mice with PB1-VAC and restimulating in vitro with influenza virus A/PR/8/34-infected feeder cells (see Methods). Subsequently, we tested the MHC restriction of the T cells by using target cells expressing either the Dk or the Kk class I molecule, but not both (Fig. 1). These experiments showed that the response to PB1 was Dk-restricted; a Kk-restricted response to PB1 was not observed.

- **Identification of PB1 peptides recognized by CD8+ T cells**

The influenza virus A/PR/8/34 polymerase protein PB1 is a polypeptide of 757 amino acids (Winter & Fields, 1982) and
Dk-restricted epitope in influenza virus

Fig. 1. Identification of the MHC class I restriction pattern of PB1-specific CTL. Polyclonal effector T cells were derived as described in Methods and, after three restimulations with antigen in vitro, were used in a chromium-release assay at the indicated effector (E):target (T) ratios. Target cells were as follows: ▲, uninfected C3H.OH cells (KdDk); ●, influenza A/PR/8/34 virus-infected C3H.OH cells; ○, uninfected NA cells (KkDdLd); ■, influenza A/PR/8/34 virus-infected NA cells.

because of its large size and the lack of an established motif for Dk-presented peptides, rather than trying to predict the Dk-restricted peptide within PB1, a more systematic approach was used. Four new recombinant VV expressing overlapping fragments of PB1 were generated and, together with PB1-VAC (expressing the full-length protein), were used to infect target cells in a cytotoxicity assay (Fig. 2). The results clearly showed no recognition of the amino-terminal 300 amino acids of PB1 and used as target cells with polyclonal effectors at an effector:target ratio of 10:1 in a chromium-release assay.

Table 1. Alignment of known Dk-restricted epitopes

<table>
<thead>
<tr>
<th>Epitope sequence</th>
<th>Protein</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>RRGKTYGL</td>
<td>Gag 88–96*</td>
<td>de Bergeyck et al. (1994)</td>
</tr>
<tr>
<td>SRLRLPSL</td>
<td>MT 236–244†</td>
<td>Lukacher &amp; Wilson (1998)</td>
</tr>
<tr>
<td>ARLGKGYMF</td>
<td>PB1 349–357</td>
<td>This paper</td>
</tr>
</tbody>
</table>

* Gag is derived from an endogenous defective murine retrovirus.
† MT is the middle T oncoprotein of polyomavirus.

Fig. 2. Mapping of Dk-restricted T cell epitopes within PB1 using recombinant VV. L929 target cells (H-2k) were infected with wild-type VV, non-recombinant VV or recombinant VV expressing the indicated amino acid residues of PB1 and used as target cells with polyclonal effectors at an effector:target ratio of 10:1 in a chromium-release assay.

Fig. 3. Mapping of Dk-restricted T cell epitopes within an 80 amino acid region of PB1 using synthetic peptides. A panel of all possible 9-mer peptides spanning the region of PB1 identified to contain at least one probable T cell epitope was synthesized and the peptides were used to sensitize L929 target cells in a chromium-release assay. Polyclonal CTL were used at an effector:target ratio of 10:1 and the peptides were present during the 5 h release at a concentration of 10 μM.

Fig. 4. The effect of peptide concentration on the recognition of peptides 22 to 26 by polyclonal PB1-specific CTL. L929 target cells were incubated with polyclonal CTL at an effector:target ratio of 10:1 in the presence of synthetic peptides at the indicated concentrations in a chromium-release assay. The peptide sequences were as follows: ●, KMARLGGY; ▲, MARLGKGYM; △, ARLGKGYMF; ○, RLGKGYMF; ■, LGKGYMFES.

Although a consensus binding motif for Dk-presented peptides derived from sequencing of peptides eluted from purified Dk molecules has not been reported, the three Dk-
restricted epitopes reported in the literature (de Bergeyck et al., 1994; Lukacher & Wilson, 1998; Wilson et al., 1999) all show consensus features (Table 1) and a predicted D<sup>k</sup> peptide-binding motif has been proposed (Lukacher & Wilson, 1998). This motif consists of a basic residue at position two, another basic residue at position five and a hydrophobic residue (preferentially a leucine) at the carboxy terminus of a 9-mer peptide. Inspection of the 150 amino acid region of PB1 (defined above) for the presence of this motif suggested that all probable epitope sequences were present within a region of 79 amino acids (corresponding to residues 326 to 404 inclusively) and therefore all possible 9-mer peptides spanning this region were synthesized and tested in a cytotoxicity assay (Fig. 3).

Surprisingly, 12 of the 71 different peptides gave greater than 20% specific lysis in the assay at the single concentration of 10 µM. Moreover, the 12 peptide sequences recognized were distributed throughout the entire region, indicating that they were not simply peptides containing different parts of one epitope. The 12 peptides eliciting positive results were used at a wide range of concentrations in further lysis experiments and one peptide, amino acid sequence ARLGKGYMF (PB1 residues 349 to 357), was found to sensitize target cells at an approximately 1000-fold lower concentration than any of the other peptides. Titration of this peptide corresponds to a T cell epitope. However, there were also a number of peptides, apparently unrelated in sequence to peptide 24, that gave significant lysis at micromolar concentrations, suggesting either the existence of other epitopes within PB1 or an unusual degree of cross-reactivity.

Great care was taken not to cross-contaminate the set of 72 different peptides, but to eliminate the possibility that the results were due to contamination, peptide 24 (the optimal peptide) and peptide 35, amino acid sequence KSMKLRTQI, which gave efficient lysis at a concentration of 10 µM and is apparently unrelated in sequence to peptide 24, were resynthesized separately. Both of these new peptide preparations were recognized by polyclonal PB1-specific T cell lines in cytotoxicity assays (data not shown). The recognition of different peptides shown in Fig. 3 is therefore unlikely to be due to cross-contamination of peptides, and contamination is definitely not the explanation for recognition of both peptides 24 and 35. Additional peptides were also synthesized, based on the results shown in Fig. 3, to test for the presence of epitopes that were not 9 residues long. These peptides, amino acid sequences ARLGKGYM (peptide 24 minus the carboxy-terminal F), SKSMKLRTQI and FRNVLSIAPI, were tested in cytotoxicity assays but did not give any specific lysis at a concentration of 1 µM (data not shown). This suggested that peptide 24 is the main epitope within the region of PB1 covered by the set of overlapping peptides and also demonstrated the functional importance of the carboxy-terminal phenylalanine residue of peptide 24.

To address whether peptide 24 was the dominant epitope within the entire PB1 protein, a polyclonal PB1-specific T cell line which had only been restimulated with influenza virus was incubated in the presence or absence of peptide 24 and the cells were stained for both CD8 and intracellular IFN-γ (Fig. 5). Approximately 90% of the CD8<sup>+</sup> cells in the polyclonal line recognized peptide 24, amino acid sequence ARLGKGYMF, and responded by producing IFN-γ. This clearly showed that peptide 24 is by far the most immunodominant epitope within the PB1 polymerase protein. We confirmed that peptide 24 is presented by the D<sup>k</sup> MHC class I molecule in an IFN-γ ELISPOT assay (Table 2). P815 cells expressing D<sup>k</sup> efficiently stimulated PB1-specific effector CTL to produce IFN-γ in the presence of peptide 24, whereas P815 cells expressing K<sup>k</sup> did not.

**Dominance hierarchy of influenza virus CD8<sup>+</sup> T cell epitopes in CBA mice**

To test the significance of the newly defined D<sup>k</sup>-restricted epitope within the PB1 protein during influenza virus infection, the ELISPOT assay was used to quantify the specific response in infected CBA (H-2<sup>b</sup>) mice. At the same time, because the dominance hierarchy of the K<sup>k</sup>-restricted responses was unknown, the frequencies of cells specific for the four different epitopes presented by the K<sup>k</sup> molecule were also determined.
Table 2. IFN-γ ELISpot assay to determine the MHC restriction of peptide ARLKKGYMF

PB1-specific effector CTL (200 cells) were incubated with 10⁶ splenocyte or 10⁵ P815 transfectant feeders with or without peptide as indicated. Data from triplicate wells are presented as the mean number of spots per well.

<table>
<thead>
<tr>
<th>Peptide concn (µM)</th>
<th>H-2k splenocyte feeders</th>
<th>P815-Dk feeders</th>
<th>P815-Kk feeders</th>
</tr>
</thead>
<tbody>
<tr>
<td>No peptide</td>
<td>2</td>
<td>10</td>
<td>NT</td>
</tr>
<tr>
<td>1</td>
<td>190</td>
<td>93</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>NT</td>
<td>117</td>
<td>2</td>
</tr>
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nt, Not tested.

(Fig. 6). Groups of four mice were infected by either the intranasal or the intraperitoneal route, as described in Methods, and their spleens were removed 8 days later as a source of responding cells. The ELISpot assay results clearly showed that the PB1 epitope (amino acids 349–357) was at least as immunodominant as any of the other influenza virus epitopes defined in H-2k mice in a primary response to infection and probably the most dominant, irrespective of the route of infection. Surprisingly, an epitope from one of the surface glycoproteins (HA amino acids 259–266) was also prominent in the response and essentially no different from the NP epitope in terms of the frequencies of epitope-specific T cells (Fig. 6). The results suggested that there is no CD8⁺ T cell epitope that is clearly and singularly immunodominant over the others in the primary response to influenza virus infection of CBA mice.

**Discussion**

In this report, we provide the first identification of an influenza virus H-2Dk-restricted epitope. This epitope is situated in the polymerase protein PB1. A Dk-restricted T cell response to influenza A virus was first described over 20 years ago (Blanden et al., 1979), well before the nature of antigen presentation to CD8⁺ T cells was fully appreciated, and a cytotoxic H-2k T cell clone specific for the PB1 polymerase was reported in 1987 (Bastin et al., 1987). The PB1 epitope is only the fourth Dk-presented peptide to be reported (de Bergeyck et al., 1994; Lukacher & Wilson, 1998) and an alignment of all these epitopes is shown in Table 1. This alignment shows a highly conserved binding motif for the Dk class I molecule, consisting of arginine at position two, arginine or lysine at position five, and, as always found for murine class I molecules, a hydrophobic residue at the carboxy terminus. All four epitopes are 9-mer peptides, but other peptide lengths may also be possible. The binding motif was predicted previously based on the amino acid sequence of the Dk molecule and modelling of a structure by comparison with other known class I structures (Lukacher & Wilson, 1998). Identification of the PB1 epitope using a systematic approach, as opposed to trying to predict the epitope, clearly confirms that the predicted binding motif is correct. Peptide-binding motifs are now available for all of the different classical MHC class I molecules in the three most commonly used haplotypes of mice, b, d and k. Although Dk expression at the cell surface is much lower than that of Kk in H-2k mice (O’Neill & McKenzie, 1980; Hackett & Askonas, 1981), there are prominent Dk-restricted responses to a number of viruses, including lymphocytic choriomeningitis virus (Zinkernagel et al., 1978). The availability of a clear binding motif may assist the characterization of these responses.

The recognition of many different peptides by PB1-specific T cells (Fig. 3) was surprising. Following previous experience of mapping Kk-restricted epitopes using sets of overlapping peptides, the expected result was for a few closely grouped peptides encompassing a single epitope to be recognized. However, this was clearly not the case and although a single peptide was recognized much more efficiently than all the
others, suggesting the existence of a single epitope, multiple unrelated peptide sequences were recognized by the T cells. Although it was originally assumed that T cell receptors must be highly specific, more recent evidence has suggested that they may be cross-reactive (Brock et al., 1996; Basu et al., 2000) and indeed it has been argued that this is an essential feature of the T cell receptor (Mason, 1998). Our results support the idea that T cell receptors may be cross-reactive for different peptides and further work to characterize this cross-reactivity using PB1-specific T cell clones is in progress.

It is intriguing that in the only other Dk-restricted T-cell response studied in detail, that against polyomavirus middle T protein, similar results were observed and T cells specific for an immunodominant epitope were shown to recognize a second peptide from within the same protein (Wilson et al., 1999). This could, to some extent, reflect particular properties of the Dk class I molecule and the T cell receptors capable of recognizing it. However, even in the influenza virus model, there are examples of cross-reactivity not involving Dk. In H-2b mice, two PB2 polymerase peptides were found to be recognized by NP-specific CD8+ T cells (Anderson et al., 1992) and we have observed that the NS1-derived peptide FDRLETI causes T cells specific for the Kk NP epitope SDYEGRKL to proliferate, although not they do not sensitise target cells for lysis (K. G. Gould, unpublished data). All of these observations suggest that although there may be relatively few immunodominant epitopes in the response to a virus infection, there may be other peptide sequences within the virus that are capable of stimulating the T cells that recognize these immunodominant epitopes and therefore contribute to the overall response.

The importance of the Dk-restricted response against influenza virus was first suggested when 6 of 11 T cell hybridomas derived from influenza virus-infected H-2b mice were found to be Dk-restricted and specific for PB1 (Daly et al., 1995). We have confirmed the significance of this response after primary influenza virus infection using an IFN-γ ELISpot assay. Our results showed that the PB1 epitope is at least as prominent as any of the other influenza virus epitopes in H-2k mice defined to date. Influenza virus NP has long been thought of as the immunodominant antigen for CD8+ T cells in mice, but while this may still be the case in H-2d mice (Chen et al., 2000), the results presented here, and recently reported results obtained with H-2b mice (Belz et al., 2000), have shown that this is not always the case, particularly in primary infection. In H-2b mice, it was thought that the Dk-restricted NP 366–374 epitope is immunodominant, but identification of a new Dk-restricted epitope in the PA polymerase protein led to the demonstration that the response to this epitope is equally prominent during primary infection (Belz et al., 2000). Interestingly, the NP 366–374 epitope was still dominant in secondary responses and the PA-specific T cells did not lyse influenza virus-infected target cells efficiently in a chromium-release assay (Belz et al., 2000). Our PB1-specific CTL were efficient at lysing influenza virus-infected target cells, but it would be worthwhile to test the prominence of PB1-specific CTL after secondary infection to see if the response was less apparent. It may also be the case that the uncharacterized Dk-restricted response to the influenza virus polymerase protein PB2 (Bennink & Yewdell, 1988) is very prominent during infection of H-2b mice, but testing this hypothesis awaits the identification of the epitope in question.

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References


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