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Virus-specific CTL responses induced by an H-2K^d-restricted, motif-negative 15-mer peptide from the fusion protein of respiratory syncytial virus

Shisong Jiang,[1]† Nicola J. Borthwick,[1] Paul Morrison,[1] George F. Gao[2] and Michael W. Steward[1]

[1] Immunology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK

[2] Laboratory of Molecular Medicine, Children's Hospital, Boston MA 02115, USA

We describe 15-mer peptide P8:F92–106 from the F protein of respiratory syncytial virus (RSV) that can act as an MHC class I-restricted (H-2K^d) epitope for RSV-specific CD8$^+$ CTL. This peptide is interesting because not only is it the first murine CTL epitope to be identified in the F protein but also because it does not contain a known allele-specific motif, as all 15 amino acids appear to be required for effective presentation to CTL. In *in vitro* MHC class I refolding experiments, peptide P8:F92–106 induced complex formation with H-2K^d heavy chains and β_2-microglobulin. Immunization of BALB/c mice with P8:F92–106 resulted in the induction of peptide and RSV-specific CTL responses as well as peptide-specific proliferative responses. Following intranasal challenge with RSV, P8:F92–106-immunized mice showed a significant reduction in viral load in the lungs compared to that seen in unimmunized mice. Furthermore, passive transfer of purified CD8$^+$ lymphocytes into BALB/c *scid* mice prior to challenge with RSV also resulted in a reduction in the virus load in lungs of challenged mice. These results indicate the potential of synthetic peptide epitopes for the induction of protective immune responses against RSV infection.

Introduction

Infection with respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and hospitalization in infants between 1 and 6 months of age (Collins *et al.*, 1996) and RSV disease can also be severe in the elderly and immunocompromised. Efforts to develop a vaccine for RSV have been hampered by the results of early studies showing that formalin-inactivated RSV vaccine caused an abnormal and pathological immune response upon subsequent exposure to RSV (Kim *et al.*, 1969). It is therefore important to develop a vaccine that can overcome this and epitope-based vaccines represent one possible approach (Blaney *et al.*, 1998; Hsu *et al.*, 1998, 1999).

Studies in the mouse model of RSV have demonstrated that both CD4$^+$ and CD8$^+$ T cells are crucial for the termination of the primary infection but that these cells may also be pathogenic (Graham *et al.*, 1991). The vaccine-enhanced illness is thought to have resulted from an inappropriate immune response leading to an excess of TH2-type cytokines, particularly IL-4 and IL-5, and an influx of eosinophils (Connors *et al.*, 1994; Waris *et al.*, 1997; Graham, 1996). The attachment glycoprotein (G) of RSV has been implicated (Openshaw *et al.*, 1992; Hsu *et al.*, 1999). Furthermore, although CTL are likely to be important in both recovery and protection from RSV, the G protein does not induce detectable CTL responses (Bangham *et al.*, 1986; Srikiatkhachorn *et al.*, 1997). In contrast, the fusion protein (F) of RSV induces a TH1-type cytokine response and the development of CD8$^+$ CTL (Bangham *et al.*, 1986; Alwan *et al.*, 1993) while immunization of CD8-deficient mice or mice depleted of CD8$^+$ T cells with the F protein led to a TH2 response and eosinophilia (Srikiatkhachorn & Braciale, 1997). Thus, to avoid immunopathology, it is necessary to design vaccines that promote CD8$^+$ T cell responses: the F protein, a major target of CTL isolated during primary RSV infection in mice (Alwan *et al.*, 1993; Kulkarni *et al.*, 1993) and humans (Cherrie *et al.*, 1992), is a likely candidate.

In this study we describe a peptide from the F protein of RSV that behaves as an allele-specific CTL epitope in BALB/c mice (H-2^d). Despite being motif-negative, this peptide was not only recognized by RSV-specific CTL but, following immunization, generated virus-specific CTL responses which

Author for correspondence: Michael W. Steward.
Fax: +44 171 9272378. e-mail michael.steward@lshtm.ac.uk

† **Present address:** Laboratory of Viral Pathogenesis, Dana–Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA.

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significantly reduced the viral load in the lungs following challenge with RSV.

Methods

Peptides. Peptide synthesis was performed by the RAMPS (Rapid Multiple Peptide Synthesis; Dupont) method using Fmoc chemistry with the Rink Amide MBHA resin (CN Biosciences UK, Beeston, Notts). The purity of the peptides was assessed by reverse-phase HPLC and by mass spectrometry (Eurogentec). All peptides were greater than 85% pure and had their expected mass. The CTL epitope M2–9 (ESYIGSINNITKQSA) from the M2 protein of RSV (Kulkarni *et al*., 1993) and a chimeric peptide representing the F peptide from the fusion protein of measles virus and the M2–9 epitope (F/M2–9) (Hsu *et al*., 1999) were used as the positive control epitopes for the CTL assays. The T helper epitope SH45–60 (CEYNVFHNKTFELPRA) from the SH protein of RSV (Kulkarni *et al*., 1993) was used as a positive control epitope for lymphocyte proliferation assays. Peptide 8 and the overlapping 9-, 10-, 11-, 12-, 13- and 14-mer peptides from its sequence were synthesized using a 9050 Peptide Synthesizer (Applied Biosystems) and purified by reverse-phase HPLC to greater than 95% purity.

H-2K^d:β_2-microglobulin:peptide P8:F92–106 complex assembly. Constructs encoding amino acids 1–280 of the heavy chain of H-2K^d and amino acids 1–99 of β_2-microglobulin were cloned and expressed in the T7 promoter-based vector pGMT7 in *E. coli* (Gao *et al*., 2000) and inclusion bodies of both K^d and β_2-microglobulin were prepared. Complex refolding with or without peptide P8:F92–106 was carried out by dilution in a redox buffer in a standard MHC class I refolding assay (Garboczi *et al*., 1992). The MHC–peptide complex formed was separated by gel filtration on a Superdex 200 column (Amersham Pharmacia) and the eluted peaks analysed by SDS–PAGE.

RSV and cell lines. The A2 strain of RSV was grown and titrated on Hep-2C cells as previously described (Hsu *et al*., 1999). BALB/c fibroblasts (H-2^d) and BCH$_4$ cells, persistently infected with RSV (R. Gaddum, AFRC, Compton, UK) were used as target cells in the CTL assays. L929 cells (H-2^k) transfected with H-2L^d molecules (T1.1.1) or with H-2D^d/L^d molecules (T37.2.1) were kindly provided by Carol S. Reiss, New York University, USA. L929 cells (H-2^k) transfected with H-2K^d molecules (Lkd) were kindly provided by Jonathan Yewdell of NIH, USA.

Mice and immunization. Mice, BALB/c (H-2^d), C57BL/6 (H-2^b) and CBA (H-2^k) mice (NIMR, Mill Hill, UK) and BALB/c CB-17 *scid* mice (LSHTM, London) were either infected intranasally (i.n.) with RSV (10^6 p.f.u./100 µl/mouse) or immunized subcutaneously (s.c.) with 100 µg peptide in complete Freund's adjuvant (Difco) followed 3 weeks later by a booster of peptide in incomplete Freund's adjuvant (Difco). For lymphocyte proliferation assays, mice were immunized s.c. with P8:F92–106 or with SH45–60 twice at weekly intervals, and the spleens removed 1 week after the second immunization.

Pulmonary and splenic CTL assays. To measure primary CTL responses in the lung, mice were infected i.n. with 10^6 p.f.u. RSV in 100 µl. Lungs were removed after 7 days and homogenized in cold DMEM-5 (Life Technologies). Lymphocytes were isolated by gradient centrifugation on Histopaque-1083 (Sigma) and cultured in RPMI 1640 (Life Technologies) containing 10% FCS, 10 mM HEPES (Life Technologies), 50 µM 2-mercaptoethanol (Sigma) and antibiotics at the required concentration as effectors. For the splenic CTL assays, splenic mononuclear cells were isolated from immunized mice and restimulated *in vitro* with 0·5 M peptide for 6–7 days.

Target cells for the CTL assays were BALB/c fibroblasts and BCH4 cells. These were labelled with ^{51}Cr (200 µCi/2 × 10^7 cells; Amersham Pharmacia). Targets were pulsed with 10 µM peptides for 1 h at 37 °C. Titration experiments confirmed this to be the optimal concentration of peptide for these experiments. ^{51}Cr-labelled non-peptide-pulsed BALB/c fibroblasts were used as a control. In the experiments with C57BL/6 mice, the target cells were C57BL/6 fibroblasts either pulsed with peptides or infected with RSV 12 h before the experiment. In the experiments with CBA mice, L929 cells were used as targets. Effectors and targets were co-cultured for 6 h in a standard chromium release assay (Hsu *et al*., 1999). In all experiments shown, the spontaneous release of ^{51}Cr from target cells incubated alone was less than 20% of the total release from target cells lysed with Triton X-100. All the assays were repeated at least twice and the data presented are representative of repeated assays.

In some experiments, effector cells were incubated with 10 µg/ml of either anti-CD8 or anti-CD4 monoclonal antibodies prior to the assay. For experiments using enzyme inhibitors, ^{51}Cr-labelled target cells (10^6) were incubated for 1 h with either 10 µM lactacystin (ICN Pharmaceuticals) or with 20 or 30 µl of a protease inhibitor cocktail containing a mixture of inhibitors for the inhibition of serine, cysteine and aspartic proteases (Sigma; cat. no. P8340). The cytotoxicity assay was performed as described above. Effectors were splenocytes from P8:F92–106-immunized BALB/c mice.

Lymphocyte purification and flow cytometric analysis. Adherent cells were removed by plastic adherence. B cells and contaminating macrophages were depleted using anti-MHC class II antibody (clone T1B 120) followed by addition of rabbit anti-rat immunoglobulin-coated Dynabeads (Dynal). The T cells were further depleted of either CD4$^+$ or CD8$^+$ subsets using anti-CD4 (clone YTS 191.1; a gift from X. Xu, Institute of Molecular Medicine, Oxford, UK) or anti-CD8 (clone 53-6.7, BD Biosciences, Cowley, Oxford, UK), respectively followed by rabbit anti-rat Ig-coated Dynabeads as before. The enriched CD4$^+$ and CD8$^+$ subsets were analysed for purity by flow cytometry.

Lymphocyte proliferation assay. Splenic lymphocytes were isolated and cultured at 2 × 10^6/ml in RPMI 1640 plus 10% FCS plus antibiotics in the presence of peptide (12·5–0 µg/ml) for 5 days. Six hours before harvesting, cells were pulsed-labelled with 1 µCi per well of [^{3}H]thymidine (Amersham Pharmacia). Results are expressed as c.p.m. (c.p.m. of peptide stimulated − c.p.m. of unstimulated cells) ± SD.

ELISA. These assays were performed as previously described (Hsu *et al*., 1999). Briefly, 96-well microplates were coated with 50 µl peptide P8:F92–106 or P18:F192–206 at 5 µg/ml or with 50 µl RSV at 5 µg/ml. Sera from mice immunized with P8:F92–106 or control peptide P18:F192–206 or with RSV were added at 1:200. Peroxidase-conjugated anti-mouse Ig (H and L chains, Nordic Immunological Laboratories) was added and 0·04% *O*-phenylenediamine/0·004% hydrogen peroxidase (Sigma) used as the substrate and the absorbance at 490 nm measured. RSV neutralization titres were assessed as previously described (Hsu *et al*., 1999). The neutralizing antibody titre was determined as the reciprocal of the highest dilution of antiserum which reduced the number of plaques by 50% of the mean value observed in control wells containing serum from unimmunized mice.

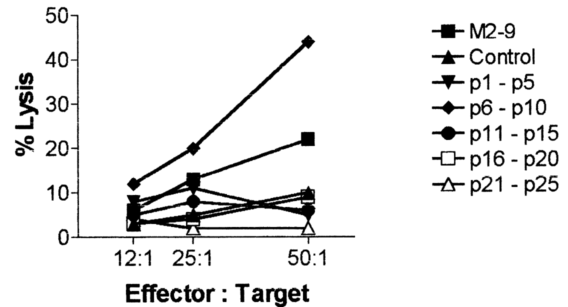
Challenge experiments. Groups of four BALB/c mice were immunized s.c. with peptides on day 0 and day 21 and were challenged i.n. with 10^6 p.f.u./100 µl RSV 3 weeks after the second immunization. Four days after challenge, the lungs were removed, weighed and homogenized. Supernatants were assayed immediately for RSV, expressed as $\log_{10}$ p.f.u./g lung. For passive transfer experiments spleno-

cytes from P8:F92–106-primed BALB/c mice were restimulated with P8:F92–106 *in vitro* for 7 days, separated into three populations – $CD8^+$ enriched, $CD4^+$ enriched and $CD8^+$ plus $CD4^+$ enriched – and transfused 10^6/mouse) into three groups of five BALB/c *scid* mice. A fourth group was reconstituted with $CD4^+$ and $CD8^+$ T cells from naïve animals. All four groups of mice and untreated mice were challenged i.n. with RSV 2 days after passive transfer of the lymphocytes. Four days later, all mice were killed and lungs removed for assessment of virus load. Means were compared using Student's *t*-test.

Results

Identification of CTL epitopes within the F protein

The amino acid sequence of the fusion protein of RSV A2 is shown in Fig. 1 (Collins *et al.*, 1984), where residues 1–21 represent the signal sequence. Starting from the 22nd residue, 55 peptides (15-mers) were synthesized, with the exception of peptide 55 which was 13 amino acids long. Each peptide overlapped the previous sequence by five residues. CTL isolated from the lungs of RSV-infected BALB/c mice were used as effector cells to screen the F protein peptides in a standard chromium release assay using BALB/c fibroblasts as targets. Initially, the peptides were split into eleven groups of five sequentially overlapping peptides. A previously described CTL epitope from the M2 protein of RSV, M2–9 (Kulkarni *et al.*, 1993), was used as a positive control, with non-pulsed BALB/c fibroblasts as a negative control. In some experiments, BCH4 cells (persistently infected with RSV) were also used. Target cells pulsed with peptides 6–10 (Fig. 2a) or with peptides 51–55 (Fig. 2b) were both efficiently killed by the RSV-specific CTL from the lung, indicating that these two groups were likely to contain one or more epitopes recognized by the CTL. Each of the peptides within groups 6–10 and 51–55 were then tested individually for CTL recognition. Only one of the peptides in group 6–10, peptide P8:F92–106, induced high levels of lysis (Fig. 3a), while in group 51–55 no single peptide gave significant CTL killing (Fig. 3b).



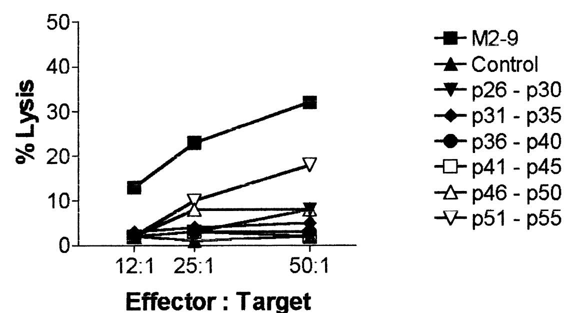


Fig. 2. CTL activity of lung lymphocytes from BALB/c mice 7 days after RSV infection versus BALB/c fibroblast targets pulsed with (a) overlapping peptide groups 1–5, p1–p25 and (b) overlapping peptide groups 6–11, p26–p55. Fibroblasts pulsed with peptide M2–9 (■) and a control 15-mer peptide (P18:F192–206) (▲) were used as positive and negative controls, respectively. Values shown represent the means of triplicate wells.

Characterization of P8:F92–106

Peptides presented by MHC class I molecules are derived from intracellular proteins that are digested by the proteasome and transported across the endoplasmic reticulum for as-

MELLILKANA ITTILTAVTF CFASGQNITE EFYQSTCSAV SKGYLSALRT
1 2 3
GWYTSVITIE LSNIKENKCN GTDAKVKLIK QELDKYKNAV TELQLLMQST
4 5 6 7 8
PPTNNRARRE LPRFMNYTLN NAKKTNVTLS KKRKRRFLGF LLGVGSAIAS
9 10 11 12 13
GVAVSKVLHL EGEVNKIKSA LLSTNKAVVS LSNGVSVLTS KVLDLKNYID
14 15 16 17 18
KQLLPIVNKQ SCSISNIETV IEFQQKNNRL LEITREFSVN AGVTTPVSTY
19 20 21 22 23
MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIM SIIKEEVLAYV
24 25 26 27 28
VQLPLYGVI DTPCWKLHTSP LCTTNTKEGS NICLTRTDRG WYCDNAGSVS
29 30 31 32 33
FFPQAETCKV QSNRVFCDTM NSLTLPSEIN LCNVDIFNPK YDCKIMTSKT
34 35 36 37 38
DVSSSVITSL GAIVSCYGKT KCTASNKNRG IIKTFSNGCD YVSNKGMDTV
39 40 41 42 43
SVGNTLYYVN KQEGKSLYVK GEPIINFYDP LVFPSDEFDA SISQVNEKIN
44 45 46 47 48
QSLAFIRKSD ELLHNVNAGK STTNIMITTI IIVIIVILLS LIAVGLLLYC
49 50 51 52 53
KARSTPVTLS KDQLSGINNI AFSN
54 55 (13mer)

Fig. 1. Amino acid sequence of the F protein of RSV (Collins *et al.*, 1984). The numbered and underlined sequences represent the overlapping peptides used in this study.

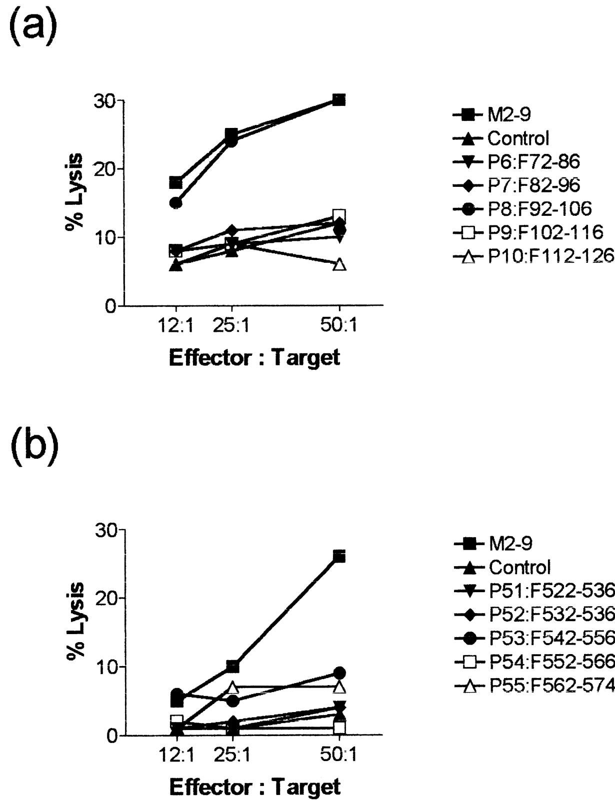


Fig. 3. CTL activity of lung lymphocytes from BALB/c mice 7 days after RSV infection versus BALB/c fibroblast targets pulsed with (a) individual peptides from peptide group 2 (peptides 6–10) and (b) peptide group11 (peptides 51–55). Fibroblasts pulsed with peptide M2–9 (■) and a control 15-mer peptide (P18:F192–206) (▲) were used as positive and negative controls, respectively. Values shown represent the means of triplicate wells.

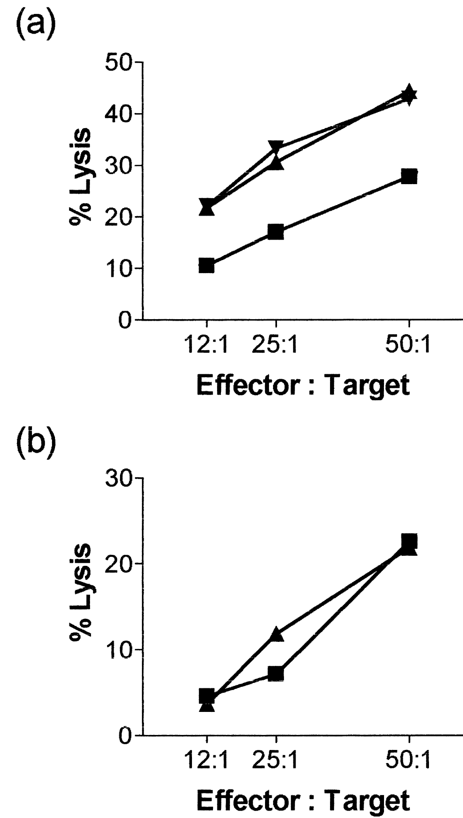


Fig. 4. (a) Effect of two concentrations of proteinase inhibitor cocktail (▲, 20 µl; ▼, 30 µl; ■, no inhibitor) on the CTL activity of splenocytes from P8:F92–106-immunized mice versus P8:F92–106-pulsed BALB/c fibroblasts. (b) Effect of the proteasome inhibitor lactacystin on the CTL activity of splenocytes from P8:F92–106-immunized BALB/c mice versus P8:F92–106-pulsed BALB/c fibroblasts. ▲, Lactacystin; ■, splenocytes alone.

Table 1. MHC restriction of CTL from BALB/c mice*

Target			%CTL lysis at E:T = 1:50	
Cell line	MHC class I expressed	P8:F92–106 pulsed	RSV-specific CTL*	P8:F92–106-specific CTL†
BALB/c fibroblast	**K^d**, D^d, L^d	+	**71·4**	**80**
BALB/c fibroblast	**K^d**, D^d, L^d	–	19·4	5·0
L929	K^b, D^b, L^b	+	13·4	14·5
L929	K^b, D^b, L^b	–	8·8	17·8
Lkd	**K^d**, K^b, D^b, L^b	+	**79·2**	**73·2**
Lkd	**K^d**, K^b, D^b, L^b	–	8·6	14·4
T1.1.1	**L^d**, K^b, D^b, L^b	+	15·2	16·4
T1.1.1	**L^d**, K^b, D^b, L^b	–	14·6	15·4
T37.2.1	**D^d**, **L^d**, K^b, D^b, L^b	+	15·3	10·1
T37.2.1	**D^d**, **L^d**, K^b, D^b, L^b	–	14·9	11·1

* Lung CTL from BALB/c mice challenged with RSV 7 days earlier.
† Splenic CTL from BALB/c mice immunized with P8:F92–106, and re-stimulated *in vitro* with P8:F92–106 in the presence of IL-2.

Table 2. Sequences of P8 peptides used to pulse BALB/c fibroblasts for lysis by RSV-specific CTL

P8:F92–106-pulsed target cells were killed by RSV-specific CTL at a range of 25–70% at an E:T ratio of 80:1 in different experiments. Lysis of target cells pulsed with all other peptides was less than 5% at an E:T of 80:1.

Peptide	Sequence
P8 (92–106)	ELQLLMQSTPPTNNR
P8-14-1 (92–105)	ELQLLMQSTPPTNN
P8-14-2 (93–106)	LQLLMQSTPPTNNR
P8-13-1 (92–104)	ELQLLMQSTPPTN
P8-13-2 (93–105)	LQLLMQSTPPTNN
P8-13-3 (94–106)	QLLMQSTPPTNNR
P8-12-1 (92–103)	ELQLLMQSTPPT
P8-12-2 (93–104)	LQLLMQSTPPTN
P8-12-3 (94–105)	QLLMQSTPPTNN
P8-12-4 (95–106)	LLMQSTPPTNNR
P8-11-1 (92–102)	ELQLLMQSTPP
P8-11-2 (93–103)	LQLLMQSTPPT
P8-11-3 (94–104)	QLLMQSTPPTN
P8-11-4 (95–105)	LLMQSTPPTNN
P8-11-5 (96–106)	LMQSTPPTNNR
P8-10-1 (92–101)	ELQLLMQSTP
P8-10-2 (93–102)	LQLLMQSTPP
P8-10-3 (94–103)	QLLMQSTPPT
P8-10-4 (95–104)	LLMQSTPPTN
P8-10-5 (96–105)	LMQSTPPTNN
P8-10-6 (97–106)	MQSTPPTNNR
P8-9-1 (92–100)	ELQLLMQST
P8-9-2 (93–101)	LQLLMQSTP
P8-9-3 (94–102)	QLLMQSTPP
P8-9-4 (95–103)	LLMQSTPPT
P8-9-5 (96–104)	LMQSTPPTN
P8-9-6 (97–105)	MQSTPPTNN
P8-9-7 (98–06)	QSTPPTNNR

sociation with MHC class I. The majority of MHC class I-associated peptides are 8–11 amino acids long. In addition, using predictive algorithms it is possible to identify peptide motifs that are likely to bind with high affinity to certain class I alleles and might therefore be assumed to be likely epitopes for $CD8^+$ CTL. Peptide P8:F92–106 is an unusual candidate for a CTL epitope, firstly because it is a 15-mer and secondly because it does not contain a motif predicted to bind to $H\text{-}2^d$ class I molecules (Engelhard, 1994). To confirm the MHC restriction of P8:F92–106, a panel of cell lines was pulsed with P8:F92–106 and used as targets in the CTL assay (Table 1). The results show that only P8:F92–106-pulsed cell lines expressing K^d molecules (BALB/c fibroblasts and Lkd cells) were capable of effectively presenting the peptide for recognition and lysis by RSV and P8:F92–106-specific BALB/c CTL. To ensure that the peptide was not undergoing proteolysis due to cell surface or media protease activity target cells were pulsed in the presence of a cocktail of protease inhibitors. There was a small, but significant, enhancement in the cytotoxic activity of the P8:F92–106-primed splenocytes against protease inhibitor-treated targets at two inhibitor concentrations (Fig. 4a). This indicates that media or cell surface protease activity may well trim the peptide but this interferes with its presentation. As a further control to ensure there was no internal processing of P8:F92–106, target cells were pulsed in the presence of lactacystin, a proteasome inhibitor (Fig. 4b). The cytotoxic activity of P8:F92–106-primed splenocytes against lactacystin-treated target cells was not significantly different to that seen with untreated target cells, indicating that the proteasome is not involved. To confirm that all 15 amino acids in P8:F92–106 are required for efficient recognition by CTL, a series of overlapping 9–15-mer peptides representing the sequence of P8:F92–106 was synthesized and tested for the ability to serve as targets. However, only the original 15-mer sequence induced lysis by RSV-specific CTL (Table 2). These data strongly suggest that the full-length 15-mer P8:F92–106 is required for recognition by CTL.

Phenotype of the RSV-specific CTL against P8:F92–106-pulsed target cells

To confirm that the P8:F92–106-specific effector cells recovered from the lungs of RSV-infected mice were class I restricted $CD8^+$ CTL, the CTL assays were performed in the presence of blocking monoclonal antibodies to either CD4 or CD8. The untreated lung lymphocytes showed high levels of killing that were reduced by treatment with either anti-CD4 or anti-CD8 antibodies (lysis at an E:T ratio of 100:1 in duplicate experiments: Untreated: 72·8, 72·0%; anti-CD8 treated: 13·4, 10·1%; anti-CD4 treated: 37·9, 30·6%). The significant reduction in CTL activity seen in anti-CD8-treated cells indicated these to be an important effector phenotype.

K^d heavy chain:β_2-microglobulin:peptide complex formation

In vitro refolding experiments with K^d heavy chain and β_2-microglobulin showed that in the presence of P8:F92–106, a complex of approximately 43 kDa was formed as assessed by Superdex 200 chromatography (Fig. 5a). This complex was shown by SDS–PAGE analysis to contain both K^d heavy chain and β_2-microglobulin (Fig. 5b). In contrast, there was no specific complex peak observed without peptide (data not shown). This indicates that the peptide is specific for K^d.

Response of BALB/c mice to immunization with P8:F92–106

To determine if immunization with P8:F92–106 alone could induce a CTL response, BALB/c mice were immunized with P8:F92–106, M2–9 (positive control) or P18:F192–206 (negative control). Splenocytes were re-stimulated *in vitro* with

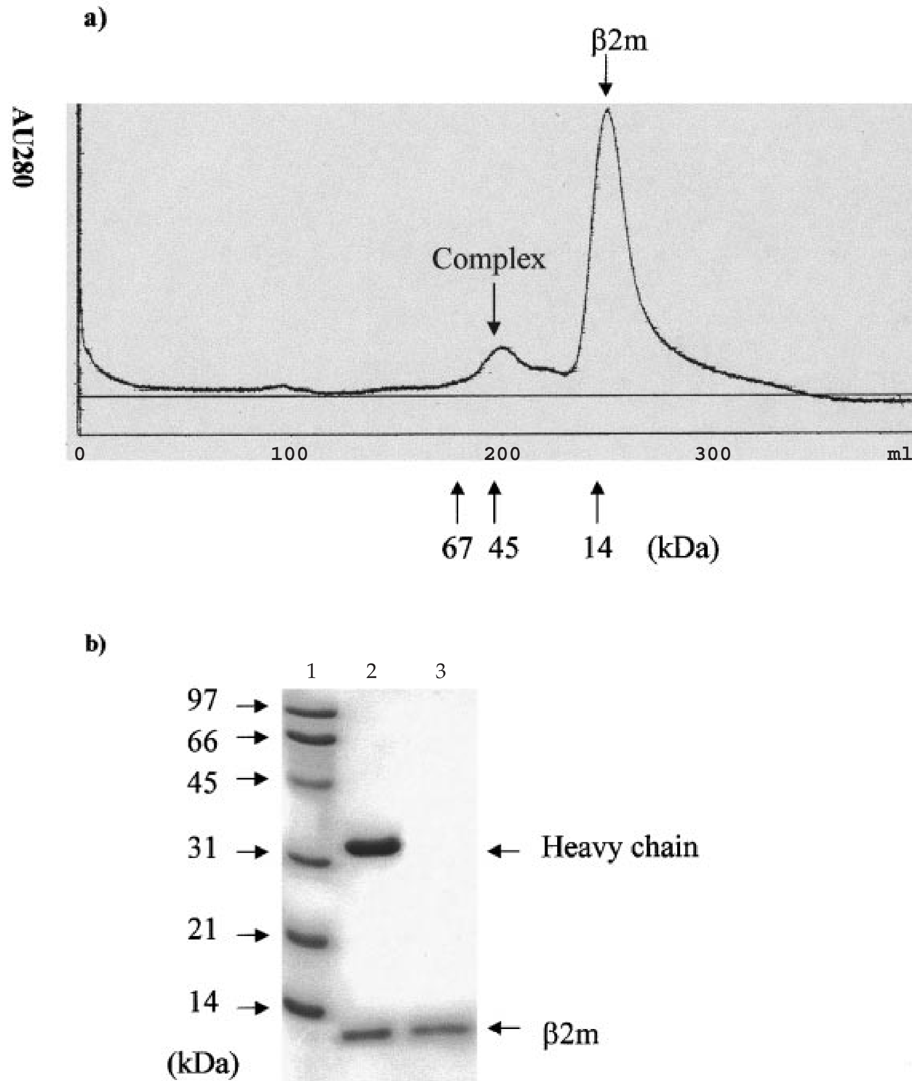


Fig. 5. (a) Gel filtration profile of the refolded K^d heavy chain:β_2-microglobulin: P8:F92–106 complex on Superdex 200. The elution positions of molecular mass standards for this column are shown. (b) SDS–PAGE analysis of the eluted peaks from the Superdex 200 separation shown in (a) stained with Coomassie blue. Lane 1 represents molecular mass standards; lane 2 represents the complex and lane 3 represents β_2-microglobulin.

the same peptides for 7 days and assayed for CTL activity. P8:F92–106-primed BALB/c lymphocytes lysed not only P8:F92–106-pulsed target cells but also the persistently RSV-infected cell line BCH4, but did not lyse cells pulsed with an irrelevant peptide (Fig. 6a). The level of lysis of BCH4 by P8:F92–106-specific CTL was similar to that seen with the CTL induced by immunization with the M2–9 peptide (Fig. 6b). Mice immunized with an irrelevant peptide, P18:F192–206, had no demonstrable CTL activity against BCH4, or P8:F92–106-pulsed targets (data not shown). To investigate the phenotype of the effector cells, splenocytes from P8:F92–106-immunized BALB/c mice were separated into $CD4^+$- and $CD8^+$-enriched populations and used as effectors in a CTL assay against either RSV-infected BCH4 cells or peptide P8:F92–106-pulsed BALB/c fibroblasts. The results clearly show that the majority of the CTL activity observed was present in the $CD8^+$ T cell subset (Fig. 6c), indicating that the peptide promotes the differentiation of classical $CD8^+$, MHC class I-restricted CTL.

Splenocytes from groups of C57BL/6 (H-2^b) and CBA (H-2^k) mice immunized with P8:F92–106 had no CTL activity against either P8:F92–106-pulsed or RSV-infected C57BL/6 fibroblasts or L929 target cells, respectively. Furthermore, splenocytes from P8:F92–106-immunized C57BL/6 and CBA mice had no demonstrable CTL activity against P8:F92–106-pulsed targets or against RSV-infected BCH4 cells (data not shown), further suggesting that P8:F92–106 is restricted to H-2^d.

Immunization of mice with peptide P8:F92–106 was also able to prime lymphocytes for a dose-dependent proliferative

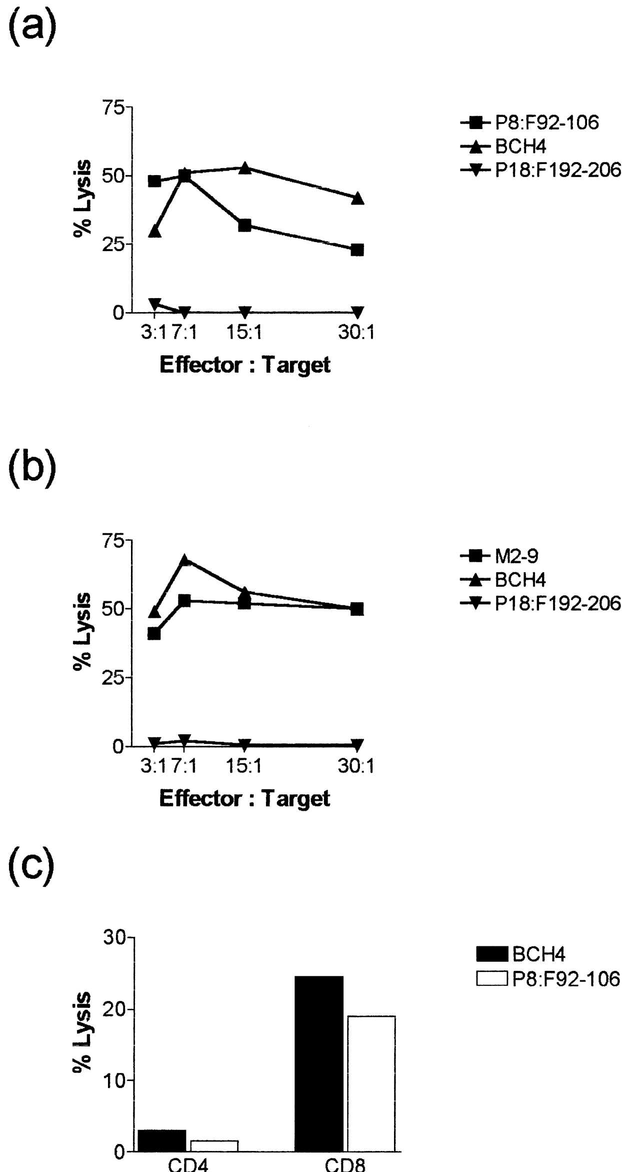


Fig. 6. CTL activity of splenocytes from BALB/c mice immunized with (a) peptide P8:F92–106 or b) peptide F/M2–9. ^{51}Cr-labelled BALB/c fibroblasts pulsed with either P8:F92–106, M2–9 or P18:F192–206 and ^{51}Cr-labelled BCH4 cells persistently infected with RSV were used as targets. (c) Phenotype of effector cells mediating the P8:F92–106- and RSV-specific CTL activity in P8:F92–106-immunized BALB/c mice. Splenocytes were separated into CD4^{+}- and CD8^{+}-enriched populations and their CTL activity versus ^{51}Cr-labelled BALB/c fibroblasts pulsed with P8:F92–106 and ^{51}Cr-labelled BCH4 cells was determined. E:T 30:1.

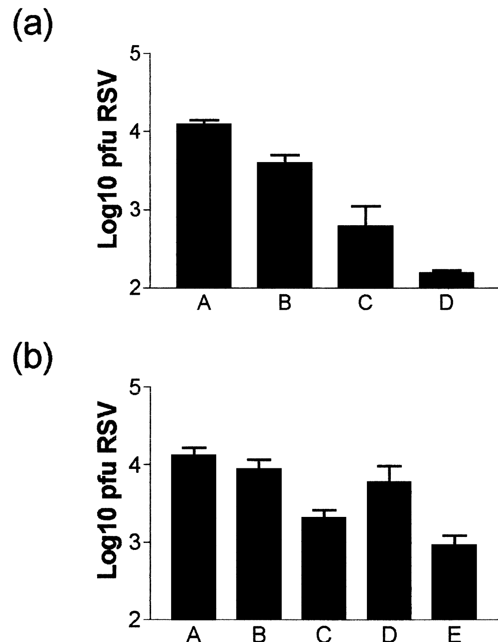


Fig. 7. (a) Virus recovered from the lungs of BALB/c mice 4 days after i.n. challenge with RSV. Column A, adjuvant only; B, P18:F192–206; C, P8:F92–106; D, F/M2–9. Data shown are $\log_{10}$ p.f.u. RSV/g of lung tissue, starting from 10^2, the lowest sensitivity of the assay. The experiment was carried out twice and similar results were obtained in each case. Values are mean $\pm$ SEM, $n = 4$. (b) Recovery of virus from the lungs of RSV-challenged BALB/c *scid* mice following passive transfer of purified lymphocytes. Column A, untreated cells; B, naïve CD4^{+} plus CD8^{+}; C, CD8^{+} cells from P8:F92–106-immunized mice; D, CD4^{+} cells from P8:F92–106-immunized mice; E, CD4^{+} plus CD8^{+} cells from P8:F92–106-immunized mice. Data shown are $\log_{10}$ p.f.u. RSV/g of lung tissue, mean $\pm$ SEM.

response upon subsequent restimulation *in vitro* (data not shown). The levels of proliferation seen were similar to those induced by the positive control, peptide SH-45, an H-2^{d} T helper cell epitope from the SH protein of RSV (Kulkarni *et al.*, 1993). In ELISA, immune serum from P8:F92–106-immunized mice was able to recognize P8:F92–106 but not RSV (A_{490} at 1/200 dilution of sera: P8:F92–106 2·00 0·11, RSV 0·16 0·03, P18:F192–206 0·20 0·01). These anti-P8:F92–106 antibodies were, however, unable to neutralize RSV *in vitro* (neutralizing titre, $\log_2$ anti-RSV 6·5 2·0, anti-P8:F92–106 0·7 0·05, anti-P18:F192–206 0·6 0·04). Thus, although immunization with P8:F92–106 induced peptide-specific antibodies, it is very unlikely that this peptide represents an important protective B cell epitope.

Effect of P8:F92–106 immunization on virus recovery following challenge with RSV

To investigate if immunization with P8:F92–106 could reduce viral load following infection with RSV, groups of mice were immunized with either P8:F92–106, F/M2–9, P18:F192–206 or with adjuvant only and then challenged with RSV. Titres from mice immunized with P8:F92–106 and the positive control F/M2–9 were 630 and 160 p.f.u./g respectively, near the limit of detection of the assay (10^2 p.f.u./g tissue). These were both significantly lower than those recovered from control, adjuvant only immunized mice (12500 p.f.u./g) or P18:F192–206-immunized mice (4000 p.f.u./g; $P < 0·001$; Fig. 7a). Thus, immunization with P8:F92–106 can significantly reduce virus titres in the lung following RSV challenge.

To confirm the role of CD8^{+} T cells as the effector cells responsible for the decrease in viral load, splenocytes from

P8:F92–106-immunized mice were purified by negative depletion into three populations: CD4$^+$ T cells, CD8$^+$ T cells and CD4$^+$ plus CD8$^+$ T cells. These cell populations were transferred into BALB/c *scid* mice: 10^6 cells were transferred into each mouse and, as a control, 10^6 T cells isolated from naïve animals were transferred into a fourth group of animals. Two days after transfer, the mice were challenged i.n. with RSV. The lungs were removed 4 days later and the RSV titre measured by plaque assay. Both the unreconstituted *scid* mice and those animals receiving T cells from naïve mice showed similar high titres of RSV in their lungs (Fig. 7b). However, the passive transfer of CD4$^+$ plus CD8$^+$ T cells and CD8$^+$ T cells alone from P8:F92–106 immune mice decreased the virus titre compared to that in the lungs of control mice ($P < 0{\cdot}001$) whilst the transfer of CD4$^+$ cells had a lesser effect (Fig. 7b).

Discussion

An optimal vaccine against RSV should include determinants for the induction of CD8$^+$ CTL. It has been demonstrated in a number of viral infections that CD8$^+$, MHC class I-restricted CTL are essential for the elimination of virus, and that the absence of these cells leads to a chronic or persistent infection. For example, CD8$^+$ T cells are responsible for the clearance of lymphocytic choriomeningitis virus (LCMV) and are the most dominant anti-viral response in this infection (Moskophidis *et al*., 1987; Fung-Leung *et al*., 1991). In RSV infection, CTL can be demonstrated within the lungs of BALB/c mice following i.n. infection (Taylor *et al*., 1985), mainly directed against the F protein with some recognizing the nucleoprotein (N) but not the G or the small hydrophobic protein, 1A (Pemberton *et al*., 1987). CD8$^+$ T cell responses have additionally been shown to play an important role in the prevention of immunopathology associated with TH2-type responses through the release of IFN-γ. The F protein of RSV was selected as a likely candidate in this study because high levels of F-specific killing can be detected in both mice and humans. In addition, levels of F-specific CTL activity are correlated with recent RSV infection in humans while in mice immunization with purified F protein significantly inhibits virus replication.

The lymphocytes used to screen for peptide-specific CTL responses were isolated from the lungs of mice during primary RSV infection. This is a useful approach because it allows the investigation of effector cells that have differentiated *in vivo* in response to the virus and migrated back into the tissue. The recognition of P8:F92–106 by these CTL indicates that this peptide must be processed and presented naturally during RSV infection resulting in CTL proliferation and differentiation. The high levels of killing suggest it is likely to be an important epitope in these animals. Although P8:F92–106-specific CD4$^+$ and CD8$^+$ T cells were isolated from the lungs, the blocking studies suggest that the main CTL activity was contained within the CD8$^+$ subset. The decreased CTL activity seen following blocking with anti-CD4 antibody may have been a non-specific effect although further studies would be needed to confirm this. CTL that are CD4$^+$ and MHC class II restricted have been described in a number of viral infections but these are usually only detected after culture *in vitro*, and are not detected if cells are assayed directly during acute infections (Enssle & Fleischer, 1990). In RSV infection, spleen cells from mice infected with live RSV and restimulated for only one cycle *in vitro* have CD8$^+$ CTL but repeated re-stimulation *in vitro* leads to the generation and outgrowth of CD4$^+$ CTL (Nicholas *et al*., 1990).

Immunization with P8:F92–106 alone resulted in CTL responses against both whole RSV and peptide-pulsed cells, confirming that P8:F92–106 is processed and presented by infected cells. The CTL from immunized mice were again found to be CD8$^+$ and these were able to reduce viral load in the lungs following RSV challenge. Immunization with a single CTL epitope has previously been shown to induce protection against viral infection or tumour growth (Feltkamp *et al*., 1993; Blaney *et al*., 1998). In some instances, protective responses were only induced following immunization with the CTL epitope linked to a T helper epitope or other carrier (Partidos *et al*., 1996). The differentiation of CTL from P8:F92–106-immunized mice and the induction of P8:F92–106-specific proliferative responses following *in vitro* restimulation strongly suggests that although the peptide does not contain a motif for binding to either MHC class I or class II, it does have epitopes for the generation of both CTL activity and T cell help. This dual function of the peptide makes it particularly interesting.

It is known that MHC class I molecules are capable of binding several different peptides. These peptides are generally 8–9 amino acids long and both N and C termini are bound by hydrogen bonds to conserved residues in the class I peptide binding cleft. In addition, two side-chains, one at the C terminus (usually position 9) and the other at the amino terminus (usually position 2), hold the peptide in allele-specific pockets (Falk *et al*., 1990, 1991). Using this information for a class I allelic product, it is possible to predict CTL epitopes from a protein sequence. A comparison of the sequence of peptide P8 (ELQLLMQSTPPTNNR) with the published H-2K^d anchor motif (1X345678X′, where X = Y, F; X′ = I, L) shows that this peptide is motif negative and reliance on motif prediction would have missed this epitope. There are several examples in the literature of other motif-negative CTL epitopes (Kast *et al*., 1993, 1994). Interestingly, peptide P7, with the sequence ELDK**Y**KNAVTE**L**QLL, has an appropriate H-2^d anchor motif but did not prime targets for lysis by RSV-specific CTL.

Peptides presented by MHC class I are produced in the cytosol by the cleavage of partially digested protein antigens by the proteasome enzyme complex. *In vivo* experiments using purified 20S proteasome have shown that this complex preferentially cuts protein antigens into peptides of either 8–9

amino acids, the usual size of MHC class I epitopes, or 14–15 amino acids, the length of P8:F92–106 (Niedermann *et al.*, 1996). An alternative explanation is that P8:F92–106 was broken down into smaller fragments ($>$ 9-mers) by protease/s that can only cleave the 15-mer sequence. This is unlikely, given that lactacystin had no effect on CTL recognition of P8:F92–106-pulsed targets. The observation that the use of a protease inhibitor cocktail (including lysosomal protease inhibitors) actually enhanced CTL recognition suggests that P8:F92–106 is not cleaved and loaded onto MHC class I by an alternative lysosomal pathway as has been shown for some measles virus fusion protein epitopes. Minimal CTL epitopes of up to13 amino acids have been reported (Urban *et al.*, 1994). Since the MHC class I binding groove is closed at both ends and can only accommodate 8–10 amino acids in a linear form, it is considered that longer peptide epitopes are accommodated by the formation of a central loop or kink in the sequence (Fremont *et al.*, 1992; Gromme *et al.*, 1999) or even by zig-zagging (Madden *et al.*, 1993). It has also been suggested that longer peptides can be accommodated by a protrusion mechanism (Stryhn *et al.*, 2000). Other work has shown that the binding of longer peptides is restricted to peptides extended at their C terminus (Horig *et al.*, 1999). The demonstration that P8:F92–106 can promote the *in vitro* folding of K^d heavy chain and β_2-microglobulin suggests that the peptide is indeed specific for K^d.

Immunization with the 15-mer peptide P8:F92–106 from the F protein of RSV results in the production of peptide- and virus-specific CTL and peptide-specific proliferative responses. Viral loads in the lungs of RSV-challenged, P8:F92–106-immunized mice were reduced compared to controls. Viral loads in BALB/c *scid* mice receiving passively transferred purified $CD8^+$ lymphocytes from P8:F92–106-immunized mice were reduced compared to controls and these data suggest that P8:F92–106 had induced protective $CD8^+$ responses. It thus seems possible that appropriately designed epitope-specific peptide vaccines could be considered for use against RSV.

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