Synergistic effect of immunization with a peptide cocktail inducing antibody, helper and cytotoxic T-cell responses on protection against respiratory syncytial virus

S.-C. Hsu, D. Chargelegue,† O. E. Obeid‡ and M. W. Steward

Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

Respiratory syncytial virus (RSV)-specific cytotoxic T lymphocytes (CTL) or neutralizing antibodies can protect against RSV infection when induced separately by immunization with synthetic peptides. In the work described here, RSV-specific neutralizing antibodies and CTLs were induced after immunization with a cocktail of peptides consisting of a B-cell mimotope (S1S-MAP), a T-helper epitope (SH:45–60) and a CTL epitope linked to a fusion (F) peptide (F/M2:81–95) that were comparable to those induced by the peptides alone. Following challenge, a 190-fold reduction in RSV titre was observed in the lungs of peptide cocktail-immunized mice. The combination of RSV-specific humoral and cellular immunity induced by the peptide cocktail was thus more effective at clearing RSV than peptide-induced humoral or cellular immunity alone.

Introduction

Respiratory syncytial virus (RSV) is a major lower respiratory tract pathogen that causes bronchiolitis and pneumonia in infants and young children and is responsible for 65 million infections and 1 million deaths every year (Hall, 1994; Heilman, 1990). Previous attempts to develop a formalin-inactivated vaccine not only failed to protect but also resulted in exacerbated disease during a subsequent RSV epidemic (Chin et al., 1969; Fulginiti et al., 1969). New attenuated vaccines have been shown to be effective in animal models (Crowe et al., 1994; Wertz & Sullender, 1992) but were either poorly immunogenic (overattenuated) or genetically unstable. Therefore, there is an urgent requirement for the development of alternative vaccines against RSV that do not induce vaccine-enhanced illness.

Passive intravenous immunization with RSV-specific neutralizing antibodies can prevent infection in the lungs (Groothuis et al., 1993; Prince et al., 1985) and passive transfer of RSV-specific T cells can clear virus effectively from the lungs of infected mice (Cannon et al., 1987). Active immunization of BALB/c mice with a peptide mimic (mimotope) of a conformational epitope from the fusion (F) protein of RSV, presented as a multiple antigen peptide (MAP), resulted in a significant reduction in virus load following challenge with RSV in vivo (Chargelegue et al., 1998). In addition, immunization with a synthetic peptide representing a cytotoxic T lymphocyte (CTL) epitope in the M2 protein of RSV covalently linked to an F peptide also resulted in a significant reduction in virus load (Hsu et al., 1998). However, the reduction in virus load in peptide-immunized mice, although significant, was not as great as that seen in RSV-immunized mice. Although the individual humoral and cellular components of the immune response to RSV have been studied, our understanding of what constitutes protective immunity is still limited (Graham et al., 1991). Since immunization with RSV induced both virus-specific humoral and cellular immunity, it is possible that the combination of these two components of the immune response may explain the enhanced protection observed with virus-induced immunity compared with that seen with peptide-induced antibody or CTLs alone. However, the induction of protective immunity is very likely to be more complex than the simple combination of humoral and cellular responses that can be assessed by in vitro techniques.

The work described in this paper was performed to determine whether or not immunization with a cocktail of synthetic peptides that induce RSV-specific humoral and cellular immune responses could result in enhanced protective immunity against RSV infection compared with those induced...
following immunization with peptides representing the B-cell and CTL epitopes alone.

**Methods**

#### Peptide synthesis

(i) **Mimotope (S1S-MAP, B-cell epitope).** A peptide mimic of a conformational epitope of RSV F protein recognized by a protective monoclonal antibody (Mab19) has been identified (HWSISKPQ) and synthesized as a tetrameric MAP construct (S1S-MAP) using Fmoc clonal antibody (Mab19) has been identified (HWSISKPQ) and synthesized as a tetrameric MAP construct (S1S-MAP) using Fmoc chemistry and TGA resin (Novabiochem) (Chargelegue et al., 1998). The purity of S1S-MAP was assessed by C18 and C8 reverse phase HPLC and by amino acid analysis.

(ii) **T-helper (Th) and CTL epitopes.** The following T-cell epitopic peptides were used: (a) a Th peptide (SH:45–60) derived from the RSV SH protein (aa 45–60) (Kulkarni et al., 1995); (b) a CTL peptide (F/M2:81–95) consisting of a chimera between a CTL epitope from the M2 protein of RSV (aa 81–95) and a fusion peptide from the F1 protein of measles virus (aa 113–131) (Hsu et al., 1998). These epitope sequences were synthesized by automated solid-phase synthesis (9050 Pep synthesizer, Milligen) by using Fmoc chemistry and TGA resin (Novabiochem). The purity of the peptides was assessed by reverse-phase HPLC and mass spectrometry.

#### Virus

RSV (A2 strain) stock was grown in HEL2 cells. The titre of RSV stock was estimated by a plaque assay and expressed as the log10 of the reciprocal of the dilution giving 1 p.f.u.

#### Animals, immunization schedules and RSV challenge

Inbred, female BALB/c (H-2b) mice were purchased from the Medical Research Council (London, UK) and used at 6–8 weeks of age.

The optimum dose of MAP for the induction of antibody responses was found to be 25 µg per mouse, as in previous studies (Chargelegue et al., 1998). Groups of seven BALB/c mice were co-immunized subcutaneously (s.c.) with 25 µg B-cell peptide, 8 µg Th peptide and/or 15 µg CTL peptide (molar ratio 1:1:1) nmol) in Freund’s incomplete adjuvant (IFA). A control group of mice was injected with IFA alone. The mice were boosted 3 and 8 weeks later with the same doses of peptides in IFA or with IFA alone and sera were collected weekly.

Groups of four mice immunized as above with either Th peptide, B-cell peptide plus Th peptide, CTL peptide plus Th peptide or B-cell peptide, Th peptide and CTL peptide (triple cocktail) were challenged intranasally (i.n.) with RSV (106 p.f.u. in 50 µl) 3–4 weeks after the second boost. A group of unimmunized mice was infected i.n. with RSV (106 p.f.u.) 9 days prior to the challenge as a control.

Four days after the challenge, the mice were killed and lungs were removed. RSV titres were estimated by a plaque assay and expressed as log10 p.f.u./g lung tissue (Chargelegue et al., 1998; Hsu et al., 1998). RSV plaques were confirmed, after methanol fixation, by immunostaining with a polyclonal anti-RSV peroxidase conjugate (Biogenesis).

#### RSV ELISA

RSV was purified by sucrose-gradient centrifugation. Immuno IV 96-well microplates were coated with 50 µg RSV at 5 µg/ml (total protein concentration) in PBS, washed with water, blocked with PBS plus 2.5% BSA for 2 h at 37 °C and washed with PBS–Tween. The plates were incubated overnight at 4 °C with 2-fold serial dilutions of polyclonal sera, washed and incubated with a rabbit anti-mouse IgG (H+L) peroxidase conjugate (Nordic) at 1:1000 in diluting buffer for 1 h at 37 °C. IgG titres are expressed as log10 of the reciprocal of the dilution giving an A492 of 0.2.

#### Virus neutralization

RSV (50 p.f.u.) was mixed with a range of dilutions of heat-inactivated sera in MEM containing 1% foetal calf serum and 25 mM HEPES for 1 h at room temperature. The virus–serum mixtures were transferred to HEp-2 cell monolayers for 3 h at 37 °C. The virus–serum mixtures were then discarded and a 100 µl carboxymethylcellulose overlay was added. The plates were observed daily for cytopathic effect and plaques were counted up to 4 days later. Neutralization titres are expressed as log10 of the reciprocal of the serum dilution that reduced the number of plaques to 50% of the mean value obtained with serum from unimmunized mice.

#### CTL assays

Three weeks after the final immunization, spleen cells were harvested and restimulated *in vitro* with 0.5 µM CTL peptide as described previously (Chargelegue et al., 1998). After 7 days, CTL activity was assessed by using CTL peptide-pulsed BALB/c fibroblasts and BCH4 cells (BALB/c fibroblasts persistently infected with RSV) as target cells.

### Results

#### Antibody responses

After immunization with a triple cocktail consisting of the RSV B-cell peptide, the Th peptide and the CTL peptide, BALB/c mice produced levels of anti-RSV antibodies and

<table>
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<th>Immunization</th>
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<th>Anti-RSV IgG titre (log10)</th>
<th>RSV neutralization (log10)</th>
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<td>&lt;1.90</td>
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<tr>
<td>Th peptide</td>
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<td>&lt;1.90</td>
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<tr>
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<td>&lt;1.90</td>
<td>&lt;3.32</td>
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</table>
Synthetic peptide vaccine against RSV

**Fig. 1.** Induction of peptide- and virus-specific CTL activity. BALB/c mice immunized s.c. with triple cocktail (a), CTL peptide plus Th peptide (b) or B-cell peptide plus Th peptide (c) and boosted 3 and 8 weeks later. CTL activity was measured 3 weeks after the last boost. Peptide-specific CTL activity was assessed with BALB/c fibroblasts pulsed with 5 µM M2:82–90 and virus-specific CTL activity was assessed with BCH4 cells (BALB/c fibroblasts persistently infected with RSV). △, M2-9-pulsed fibroblasts; ○, RSV-infected fibroblasts; ●, control fibroblasts. E:T, effector:target.

RSV-neutralizing antibodies that were not significantly different from those obtained following co-immunization with the B-cell peptide and the Th peptide (Table 1). In addition, the titres obtained were similar to those observed after immunization with RSV (Hsu et al., 1998). Anti-RSV antibodies were not detected in groups of mice immunized with the B-cell peptide alone, with the Th peptide alone, with the Th peptide plus the CTL peptide or with adjuvant alone.

**CTL responses**

Peptide- and virus-specific CTL responses were induced following immunization of BALB/c mice with the triple cocktail (Fig. 1a) and with a mixture of the CTL and Th peptides (Fig. 1b). However, the percentage lysis induced by spleen cells from mice immunized with the triple cocktail was greater than that induced by spleen cells from mice immunized with the CTL and Th peptides. No specific CTL activity was observed in mice immunized with a mixture of the B-cell and the Th peptides (Fig. 1c).

**Protection studies**

Virus load in the lungs of immunized BALB/c mice was assessed 4 days after i.n. challenge with RSV. The lowest level of virus detectable in this assay was 1–7 log_{10} p.f.u./g lung tissue (approx. 5 p.f.u. per animal). A significant reduction in virus load was observed in mice immunized either with B-cell peptide plus Th peptide or with CTL peptide plus Th peptide compared with the virus load seen in mice immunized with Th peptide alone or in unimmunized, challenged mice (P < 0.001; Fig. 2). However, after immunization with the cocktail consisting of all three peptides, a significantly increased clearance of virus was observed, corresponding to a reduction of 2–28 log_{10} p.f.u./g (equivalent to 190-fold) compared with the controls. In two of the four animals in this group, no virus could be detected. No reduction in virus load was observed in mice immunized with Th peptide alone compared with that in unimmunized, control animals.
Discussion

RSV-specific CTL responses induced by immunization with synthetic peptides or recombinant vaccinia virus directed towards a single epitope (M2-9) can reduce virus load in the lungs of RSV-infected mice (Chargelegue et al., 1998; Kulkarni et al., 1995). A peptide mimic (mimotope) of a protective, conformation-dependent B-cell epitope from the F protein of the virus, presented as a MAP construct (S1S-MAP), induced virus-neutralizing antibodies and reduced virus load in vivo (Chargelegue et al., 1998). These observations indicate that immune responses directed towards either a CTL epitope or a B-cell epitope are capable of reducing virus load in the absence of a demonstrable response in the alternative arm of the immune response. In the work described here, we sought to determine whether peptide-induced humoral or cell-mediated responses would act synergistically to enhance virus clearance compared with that seen with antibody or CTL responses alone. Immunization with a cocktail of synthetic peptides, consisting of the B-cell peptide, the Th peptide and the CTL peptide, induced both humoral and cellular immune responses and resulted in a greater reduction in virus load in the lungs of RSV-challenged animals than that seen in mice immunized with either Th peptide plus CTL peptide or B-cell peptide plus Th peptide (Table 1; Figs 1 and 2).

The titres of anti-RSV IgG and RSV-neutralizing antibodies elicited by the peptide cocktail were similar to those raised after immunization with Th peptide plus B-cell peptide. Furthermore, these results suggest that the priming of RSV-specific CTL responses did not alter the development of RSV-specific antibody responses (Table 1; Fig. 1). However, RSV-specific CTL responses induced by the triple cocktail were greater than those induced after immunization with Th and CTL peptides. These enhanced CTL responses may result, at least in part, from cytokines induced after immunization with the triple cocktail.

The reduction in RSV recoverable from the lungs of peptide cocktail-immunized mice after virus challenge was similar to that observed in RSV-immunized animals and was significantly greater than that seen in mice immunized with B-cell peptide plus Th peptide or CTL peptide (Fig. 2). These results are consistent with reports of other authors, in which immunization with F protein formulated into immune-stimulating complexes or with vaccinia virus expressing F protein induced both humoral and cellular immune responses and resulted in reductions in virus titre of more than 2 log<sub>10</sub> (Connors et al., 1991; Trudel et al., 1992).

Immunization with purified F protein or with formalin-inactivated RSV resulted in enhanced pulmonary pathology following RSV challenge (Trudel et al., 1992; Walsh, 1994) by mechanisms involving CD4<sup>+</sup> T cells (Connors et al., 1992; Alwan et al., 1992). Furthermore, priming with the attachment glycoprotein (G) of RSV resulted in eosinophilia and atypical lung disease (Openshaw et al., 1992), again mediated by CD4<sup>+</sup> T cells. Pulmonary eosinophilia and enhanced illness induced by soluble proteins or formalin-inactivated RSV could be due to a failure to elicit virus-specific CD8<sup>+</sup> T-cell activity (Srikitakbachorn & Braciale, 1997; Alwan et al., 1992). Immunization with whole proteins or with inactivated vaccines may not always result in the induction of appropriate responses, since these vaccine preparations may not be formulated in a way to allow antigen processing and presentation via the MHC class I pathway. Antigenic variations within a single epitope may allow mutant viruses to escape immune responses directed towards that epitope; it will therefore be necessary to immunize with more than one epitope to overcome the emergence of mutants.

Epitope-based vaccines may be an effective vaccine strategy for the induction of protective immune responses in an optimal micro-environment whilst avoiding unwanted responses induced by potentially pathogenic regions of RSV antigens (Sparer et al., 1998). Although mutated proteins in which the pathogenic region is deleted could induce protective immunity against RSV without enhanced pathogenicity, antigen conformation or presentation could be altered and important protective B-cell epitopes may be missing (Gaddum et al., 1996). Different genetic, physical or chemical modifications of subunit proteins have been used to improve CTL induction after immunization and, although these approaches have led to enhanced humoral and cellular immune responses, they appeared to enhance one arm of the immune response whilst inhibiting the other (Rodriguez et al., 1997; Speidel et al., 1997).

Although it is clear from several studies that virus neutralization in vitro by anti-virus antibodies does not always correlate with protection in vivo (Bachmann et al., 1997), neutralizing antibodies can block the entry of virus into cells and represent one of the possible protective mechanisms. However, the presence of antibodies alone may not be sufficient to abort the spread of RSV by a fusion process between virus-infected and healthy cells, although antibodies may interfere with virus multiplication intracellularly by antibody-dependent cytotoxicity or complement-dependent lysis. On the other hand, virus-specific CTLs can abort intracellular virus replication efficiently or can block the entry of virus into cells, either by cytotoxicity or by the generation of non-cytotoxic, soluble mediators. RSV-specific CTLs may represent an efficient effector mechanism by which the host interferes with the propagation of RSV by fusion. The synergistic effect on protection against RSV observed in the present study after immunization with peptides inducing antibody and CTL responses might result from the production of appropriate cytokines or from other, as yet unknown, cooperative mechanisms between humoral and cellular immune responses.

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References


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