Protective cytotoxic T lymphocyte responses against paramyxoviruses induced by epitope-based DNA vaccines: involvement of IFN-γ

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Abstract
Plasmid DNA vectors have been constructed with minigenes encoding a single cytotoxic T lymphocyte (CTL) epitope from either the M2 protein of respiratory syncytial virus (RSV) or from the nucleoprotein of measles virus (MV) with or without a signal sequence (also called secretory or leader sequence). Following intradermal immunization, plasmids in which the CTL epitopes were expressed in-frame with the signal sequence were more effective at inducing peptide- and virus-specific CTL responses than plasmids expressing CTL epitopes without the signal sequence. This immunization resulted in protection against MV-induced encephalitis and a significant reduction in viral load following RSV challenge. The reduction of viral load following RSV challenge was abrogated by prior injection with anti-IFN-γ antibodies. These results highlight the ability of epitope-based DNA immunization to induce protective immune responses to well-defined epitopes and indicate the potential of this approach for the development of vaccines against infectious diseases.

Introduction
The induction of specific cytotoxic T lymphocyte (CTL) responses by epitope-based vaccines has been shown to result in protection against several infections (1,2). Epitope-based vaccines used so far have involved immunization with synthetic peptides and with epitopes expressed on live viral vectors. Peptides alone are generally poor immunogens, requiring the use of adjuvants or lipophilic modification of the peptide for the successful induction of CTL responses (3,4). Live viral vectors have the disadvantage that boosting is likely to be ineffective and they are potentially dangerous in immunocompromised hosts.

Early attempts to develop vaccines with inactivated paramyxoviruses such as measles virus (MV) and respiratory syncytial viruses (RSV) not only failed to protect, but also resulted in exacerbated disease during subsequent epidemics (5,6). Priming with the attachment glycoprotein (G) of RSV resulted in eosinophilia and the induction of atypical pulmonary disease, which are mediated by CD4+ T,2 responses (7). There is an urgent requirement for the development of vaccines against respiratory virus infections which will not cause vaccine-enhanced illness. This is particularly the case for RSV, against which there is currently no licensed vaccine. Furthermore, the immunosuppressive effect of maternal anti-MV antibodies resulting in the poor responses to MV vaccine in infants (8) highlights the need to develop vaccines which can be effective even in the presence of maternally derived antibodies. However, immunization with whole proteins or organisms may not always result in the induction of appropriate responses.

DNA vaccines consisting of plasmid DNA vectors carrying the immediate early promoter from human cytomegalovirus (CMV) and encoding the entire cloned open reading frame...
coding for whole proteins have been shown to result in the development of both humoral and cellular immunity against virus infection (9). Plasmid DNA coding for a single T cell epitope has also been shown to induce cellular immunity against a tumor (10). However, little has been done to utilize the potential of DNA vaccines to induce epitope-specific immune responses against infectious diseases. The use of DNA vaccines avoids the complicated physicochemical problems associated with the use of adjuvants and may also result in in vivo antigen presentation of the encoded epitopes in a manner similar to the presentation of the epitope that would occur following natural infection.

In the work described here, plasmid DNA vectors (pcDNA3) have been constructed which contain minigenes encoding either a single CTL epitope derived from the M2 protein of RSV or a single CTL epitope from the nucleoprotein of MV (11, 12). In addition, we have also used a vector pSecTag B which encodes the secretory sequence from the murine Ig κ chain. This secretory sequence improves the recognition of target cells by CTL and may help transport epitopes into the endoplasmic reticulum (ER) for binding to class I MHC molecules. Since recent evidence has showed that skin cells play an important role in DNA-induced CTL responses (13), the ability of these constructs to induce epitope-specific CTL responses was assessed following intradermal (i.d.) immunization. Furthermore, the constructs were also used to determine whether or not the CTL responses they induced had an in vivo protective effect in murine models of infection of MV and RSV. Since cytotoxic T cells can mediate antiviral protection via two pathways, either direct cytotoxicity or via released cytokines, such as IFN-γ, the role of this cytokine in DNA-induced immunity was also investigated.

Methods

The construction of recombinant plasmid DNAs

Synthetic oligonucleotides were designed and synthesized (Genosys, Cambridge, UK) as minigenes encoding 9 amino acids of a single CTL epitope either from the M2 protein of RSV (M2-1 and M2-2) (11) or from the nucleoprotein of MV (NP-1 and NP-2) (12) (Table 1). These minigenes were flanked by non-complementary sticky restriction sites (HindIII and NotI; Promega, Madison, WI). M2-1 (5′ → 3′) complementary to M2-2 (3′ → 5′) and NP-1 (5′ → 3′) complementary to NP-2 (3′ → 5′) were annealed and then inserted between HindIII and NotI sites into pcDNA3 or pSecTag B vectors (Invitrogen, San Diego, CA) which contain CMV immediate-early gene promoter, 11 origin of replication, simian virus 40 promoter and origin, bovine growth hormone polyadenylation site, and a multiple cloning site. The Ig κ chain leader sequence lies between the CMV promoter and the multiple cloning site of the pSecTag B vector. All inserts were sequenced after construction. Plasmid DNAs were grown in Escherichia coli JM109 and DNA concentrations were determined by optical density measurements at 260 nm.

Mice and immunization

BALB/c or CBA mice were immunized i.d., with 50 µg plasmid DNA in PBS twice on day 0 and 21 as follows: (i) pSecTag B expressing M2:82–90 (pSecTag-M2), (ii) pSecTag B expressing NP:52–60 (pSecTag-NP), (iii) pcDNA3 expressing M2:82–90 (pcDNA3-M2) and (iv) pcDNA3 expressing NP:52–60 (pcDNA3-NP). Three weeks after the last injection, mice were killed and their spleen cells were obtained for in vitro assessment of peptide- and virus-specific CTL activity.

CTL generation

Three weeks after the second immunization, four or five mice were killed, their spleens removed and mononuclear cells were re-stimulated in vitro with 0.5 µM of the appropriate peptides for 6 days, in 10 % concanavalin A supernatant containing medium (RPMI 1640 medium containing 10 % FCS, 2 mM L-glutamine, 10 µg/ml penicillin, 10 µg/ml streptomycin, 10 mM HEPES buffer and 50 µM 2-mercaptoethanol). The CTL activity of these effector cells was assessed by a 51Cr-release assay.

51Cr-release assay

The following target cells were used in cytotoxicity assays: L929 (H-2b, MHC class I−) and MHC class II+), NS-20Y, MV-infected NS-20Y (murine neuroblastoma cells persistently infected with MV) (NS-20Y-MV) (14), BALB/c fibroblasts (H-2b) and BCH4 (BALB/c fibroblast persistently infected with the long strain of RSV) were kindly provided by Dr R. Gaddum (AFRC, Compton, UK). BALB/c fibroblast and BCH4 cell lines were maintained in DMEM with 10 % FCS, 2 mM L-glutamine, 10 µg/ml penicillin, 10 µg/ml streptomycin, 10 mM HEPES buffer and 50 µM 2-mercaptoethanol. The CTL activity of these effector cells was assessed by a 51Cr-release assay.

Table 1. The amino acid and oligonucleotide sequences of CTL epitopes used

<table>
<thead>
<tr>
<th>Designation of epitope used</th>
<th>Amino acid sequences</th>
<th>Oligonucleotide sequences</th>
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<tbody>
<tr>
<td>NP:52–60</td>
<td>LDRLVRLIG</td>
<td>NP-1: AGCTTATGCTTGACGCCGATCGTACGTCAATAGGATAGGC</td>
</tr>
<tr>
<td>M2:82–90</td>
<td>SYIGSINNI</td>
<td>NP-2: ATACGAACTGGCTGCGAGCATGCGAGATTATCCTATCCGCGG</td>
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<tr>
<td></td>
<td></td>
<td>M2-1: AGCTTATGCTTGACGCCGATCGTACGTCAATAGGATAGGC</td>
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<tr>
<td></td>
<td></td>
<td>M2-2: ATACGAACTGGCTGCGAGCATGCGAGATTATCCTATCCGCGG</td>
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target cells for measuring MV-specific or RSV-specific CTL activity respectively. All target cells were pulsed with 200 μCi $^{51}$Cr for 90 min. $^{51}$Cr-labelled target cells and effector cells were co-cultured for 6 h at the indicated E:T cell ratios.

Protection studies

Challenge of mice with RSV. BALB/c mice (four or five mice per group) were i.d. immunized on day 0 and 21 with 50 μg of plasmid DNA expressing appropriate CTL epitopes from either the M2 protein of RSV or from the nucleoprotein of MV. A group of unimmunized mice was included as a control. Mice were challenged with $10^6$ p.f.u./50 μl 3 weeks after second immunization. Four days after challenge, the lungs were removed, placed in sterile stabilizing buffer (1 ml per lung) and homogenized (15). The homogenates were centrifuged, and log$_{10}$ dilutions of the supernatants were made and assayed immediately on HEp-2 cell monolayers in 24-well flat-bottomed tissue culture microwell plates. The cells were incubated for 3 h at 37°C and the inoculum was removed, the cell monolayer washed once with MEM medium and substituted with 1 ml carboxymethylcellulose medium. After 4 days, the cells were fixed and stained with peroxidase-conjugated anti-RSV antibodies and the RSV titers were expressed as log$_{10}$ p.f.u./g lung.

Antibody administration for in vivo neutralization of IFN-γ. Three groups of pSecTag-M2 immunized mice received either hamster IgG mAb specific for murine IFN-γ (H22) (provided by Dr. Gregory J. Bancroft and originally obtained from Professor R. D. Schreiber, Washington University, St Louis, MO) or an isotype control antibody L2 i.p. at 300 μg per mouse or were untreated to serve as a further control. After 24 h, all mice were infected with $10^6$ p.f.u./50 μl of RSV. Four days after infection, the lungs were harvested and RSV was titrated as described above.

Challenge of mice with neuroadapted MV. Two groups of 2-week-old CBA (H-2$k$) mice (six mice per group) were immunized i.d. with 50 μg of pSecTag-NP or pSecTag-M2 in saline and boosted 2 weeks later with the same dose in saline. An extra group of naive mice (four mice per group) was included as a further control. Mice were challenged intracranially (i.c.) 22 weeks after the boost with $10^4$ p.f.u. of neuroadapted MV per mouse in 25–30 μl volumes (16). Mice were monitored daily for the development of clinical signs of disease. The survival in immunized and control groups was assessed over a period of up to 30 days after challenge.

Statistics

The significance of differences in survival or in the reduction of virus titer following viral challenge between DNA vaccine-immunized, control vector-immunized and non-immunized mice were analyzed by the Kaplan–Meier survival curve, log-rank test and the Student’s t-test.

Results

Induction of peptide- and virus-specific CTL responses by DNA vaccines containing minigenes encoding CTL epitopes

Splenocytes from CBA mice immunized with pSecTag-NP showed greater peptide- and MV-specific CTL responses than splenocytes from mice immunized with pcDNA3-NP (Fig. 1). Immunization with pSecTag-M2 induced peptide- and RSV-specific CTL responses in BALB/c mice, but splenocytes from mice immunized with pcDNA3-M2 had no demonstrable peptide or RSV-specific CTL activity (Fig. 2).

Protection against MV-induced encephalitis

Since immunization with pSecTag-NP plasmid DNA induced MV-specific CTL responses, the ability of this construct to confer protection against MV-induced encephalitis was tested. Two groups of CBA mice were immunized with either pSecTag-NP or pSecTag-M2 as a control vector and boosted 2 weeks later. A control group of naive mice was included. Mice were challenged i.c. with $10^4$ p.f.u. MV, and the development of neurological symptoms and survival were assessed daily. Percentage survival of plasmid DNA-immunized and control animals is presented in Fig. 3. In mice immunized with pSecTag-NP plasmid DNA and challenged with MV, percentage survival was 83% at 16 days post-challenge, 67% until 24 days post-challenge and 50% at 25 days after challenge, and there was no further mortality 30 days post-challenge, whereas in mice immunized with pSecTag-M2 and in naive mice challenge with MV resulted in percentage survival values of 17 and 0% respectively by day 12 post-challenge. These values are consistent with previously-published data using this model (16). These results represent a significant delay in the mortality of pSecTag-NP-immunized mice in comparison with the control groups when analyzed by the Kaplan–Meier survival curve and log-rank test ($P = 0.02$).

Protection against RSV infection

The effect of immunization with pSecTag-M2 and pcDNA3-M2 on the response to RSV infection was assessed in BALB/c mice. A significant reduction in RSV titer was observed in the lungs of pSecTag-M2 immunized mice compared to those of control, pSecTag-NP immunized mice ($P = 0.012$). Immunization with pcDNA3-M2 did not result in a significant reduction of viral load in the lungs compared to those of controls ($P = 0.30$, Student’s t-test) (Fig. 4). The reduction of viral load in vivo therefore appears to correlate with RSV-specific CTL activity in vitro.

IFN-γ depletion

pSecTag-M2 immunized mice had significantly lower levels of RSV recoverable from their lungs following challenge than did control, non-immunized mice. The significant reduction in RSV titer was abrogated in pSecTag-M2 immunized mice treated with anti-IFN-γ antibodies but not in mice treated with control antibodies ($P = 0.012$) (Fig. 5). As a further control, it was shown that the level of virus recoverable from the lungs of pcDNA3-M-2-immunized, IFN-γ-depleted mice following RSV challenge (3.58 ± 0.58 p.f.u./g) was not significantly different from that recovered from naive mice.

Discussion

Protective immune responses have been successfully induced following immunization with attenuated or inactivated pathogens against several infectious diseases. However, attempts to vaccinate against RSV or measles virus with
CTL responses induced by epitope-based DNA vaccines

Fig. 1. Peptide- and MV-specific CTL responses following DNA immunization. CBA mice were immunized i.d. with pSecTag-NP (A), pSecTag-M2 (B), pcDNA3-NP (C) and pcDNA3-M2 (D) in PBS twice on day 0 and 21. Peptide-specific CTL responses in splenocytes 3 weeks after the second immunization were measured with L929 cells pulsed with NP:52–60 (L929-NP) and measles-specific CTL responses were measured with NS-20Y persistently infected with measles virus (NS-20Y-MV).

Tween/ether- or formalin-inactivated whole virus vaccines resulted in an enhanced illness on subsequent infection. Furthermore, natural infection with RSV does not induce sustained protective immunity (17) although the severity of illness diminishes with successive infections.

The presentation pathway of endogenously produced proteins or peptides to CTL is thought to be through proteosomal processing and TAP transport into the ER, and in this context it is important to recognize that the products of plasmid DNA constructs are endogenously synthesized inside the cells. A number of signal/secretory sequences have been shown to improve the efficiency of immunization with CTL epitopes (10,18), perhaps by the transport of the CTL epitopes into the ER through a TAP-independent pathway utilizing a membrane-bound transport protein. For some CTL epitopes, the signal sequence does not enhance CTL responses (10). It is likely that some CTL epitopes could be transported into the ER by a TAP-dependent pathway more efficiently than others and the presence of the signal sequence with these epitopes will not necessarily be beneficial.

In the work described here, immunization with pSecTag-NP induced much stronger CTL responses than did a pcDNA3 construct expressing the same epitope (Fig. 1). Surprisingly, pcDNA3-M2 did not induce detectable MV-specific CTL responses (Fig. 2). There are several possible explanations for this observation. First, certain epitopes may not be transported efficiently into the ER and, therefore, signal sequences may be required to facilitate this process to achieve the induction of CTL. Second, it has been shown that vaccinia expression of Mycobacterium tuberculosis antigens is enhanced by a eukaryotic signal sequence (19). Finally, signal sequences may also have the capacity to enhance gene expression following DNA immunization. The presence of a eukaryotic translational signal, such as the Kozak sequence (CCACC), might also enhance the expression of the M2 CTL epitope in vivo after immunization with pSecTag-M2 (20).

The results described in this paper indicate that immunization with DNA vaccines containing minigenes coding for CTL epitopes can significantly protect against two paramyxovirus infections, RSV and MV (Figs 3 and 4). In the murine model of RSV, viral load was significantly reduced by >1 log10 (P = 0.012) following immunization with pSecTag-M2. Spleen cells from pSecTag-M2 immunized mice restimulated with M2:82–90 peptide in vitro produced significant amounts of IFN-γ compared to spleen cells from control mice (data not shown). The reduction in viral load in the lungs of immunized mice was abrogated by in vivo treatment with anti-IFN-γ on the day before challenge (Fig. 5) suggesting that the clearance of RSV from the lungs by plasmid DNA induced immunity involved the production of IFN-γ. Following immunization of CBA mice with pSecTag-NP plasmid DNA,
CTL responses induced by epitope-based DNA vaccines

Fig. 2. BALB/c mice were immunized i.d. with pSecTag-M2 (A), pSecTag-NP (B), pcDNA3-M2 (C) and pcDNA3-NP (D) in PBS on day 0 and 21. Peptide-specific CTL responses in splenocytes 3 weeks after the second immunization were measured with BALB/c fibroblasts pulsed with M2:82–90 (BALB/c fibroblasts + M2). RSV-specific CTL responses were measured with BCH4 cells (BALB/c fibroblasts persistently infected with RSV).

Fig. 3. Protective effect of DNA immunization against MV-induced encephalitis. CBA mice were immunized i.d. on day 0 and 14 with 50 µg pSecTag-NP (●) or with pSecTag-M2 (×) as a control vector. Naive mice (▲) were used as controls. Mice were challenged i.c. 2 weeks after the second immunization with 10⁶ p.f.u. of a neuroadapted strain of MV. Data are representative of experiments repeated three times.

Fig. 4. Protective effect of DNA immunization on RSV infection. BALB/c mice were immunized i.d. on day 0 and 21 with 50 µg pSecTag-M2, pcDNA-M2 or pSecTag-NP as a control. Three weeks after the second immunization, mice were challenged with 10⁹ p.f.u. of RSV by the intranasal (i.n.) route. Animals in the RSV control group were immunized with RSV (10⁶ p.f.u.) on day 0 and were challenged i.n. with RSV on day 9, and those in the negative control group were challenged i.n. with RSV only. Naive mice were challenged i.n. with RSV as a further control. Four days after i.n. challenge, RSV was harvested from lungs from all mice and the titer of virus expressed as p.f.u. of RSV/g of lung. Data are representative of experiments repeated three times.
MV-specific CTL responses were induced and these resulted in a significant delay in mortality from MV-induced encephalitis. In previously published work, no protection was observed following immunization with a synthetic peptide consisting of the NP-6 epitope linked to two copies of a Th epitope, even though virus-specific CTL activity was observed (21). It has been reported that susceptibility to MV-induced encephalitis in mice is correlated to impaired antigen presentation (22) which could result in low expression of MHC molecules containing bound CTL epitope peptide on the surface of infected target cells. Furthermore, virus-specific CTL responses with a higher frequency or with higher avidity will be required to achieve significant protection against MV-induced encephalitis in vivo. Such higher frequencies or avidity of virus-specific CTL responses may be more effectively induced and maintained following plasmid DNA immunization than following immunization with synthetic peptides (Fig. 1). It is possible that DNA-induced CTL may be more efficient at producing IFN-γ than those induced by immunization with peptides and cholera toxin. The possibility exists that the inclusion of other CTL epitopes and B cell epitopes from MV proteins in the same expression system may provide appropriately diverse specificities for an enhanced level of protection against MV-induced encephalitis. Although there is an effective live, attenuated vaccine against measles, the infection still accounts for more than a million childhood deaths annually in the developing world and there is a need for the development of a stable vaccine, not requiring a cold chain, which would be effective even in the presence of maternal antibodies. An appropriately designed DNA vaccine may well meet these criteria.

Passive transfer of RSV-specific T cells has been shown to effectively clear RSV (23); however, the transfer of high doses of RSV-specific CTL resulted in enhanced illness (24). In the experiments described here, no enhanced pathology was observed in plasmid DNA-immunized mice (data not shown).

Several reports have indicated that the protective effect of CTL against cytopathic viruses is dependent on their production of cytokines such as IFN-γ, rather than upon classical cytolytic mechanisms (25). IFN-γ has also been shown to enhance class I-restricted antigen processing and presentation, and to promote cell-mediated responses (26). Although in our experiments we have not formally demonstrated that IFN-γ was produced by CD8+ CTL, the fact that the RSV CTL epitope used was H-2Kd restricted and has no Th activity makes it unlikely that other cells produced the IFN-γ. It is possible that DNA vaccine-primed CTL precursors secreted accumulated amounts of IFN-γ required to interfere with viral replication during RSV infection. IFN-γ may also interfere viral replication by short-range secretion through the cognate binding of CTL to virus-infected cells (27). Alternatively, increased numbers of CTL precursors may develop more rapidly in the Th1 cytokine environment, leading to the effective clearance of RSV.

Previous work has shown that primary CTL responses induced by vaccinia recombinants expressing the M2 CTL epitope of RSV can significantly protect against RSV challenge at 6 days after i.p. or i.n. immunization. However, protective memory CTL responses waned after 9 days and had disappeared at 28 days after immunization (28). In the work described here, memory CTL responses induced by pSecTag-M2 were still present and able to significantly reduce the peak viral titer in the lungs 3 weeks after i.d. immunization (Fig. 4). Different mechanisms have been proposed to explain the maintenance of protective memory CTL responses, including increased precursor frequencies in an antigen-independent environment (29), elevated precursor frequencies induced by recurrent infections involving cross-reactive antigens (30) or bystander effects mediated by cytokines such as IL-2 (31). The persistence of a non-retroviral RNA virus in a DNA form in the host after acute infection (32) may represent another way by which memory is maintained. Plasmid DNA and its encoded proteins have been shown to persist for 30 days in vivo following i.d. immunization (33) which could explain the results we have obtained. Protective immunity following i.d. immunization with plasmid DNA might be maintained by the class I MHC-bound CTL epitope continuously expressed by DNA constructs in vivo. Protective memory CTL pools may also be maintained by T<sub>H</sub>1 cytokines and remain active long after immunization with the plasmid DNA (34). RSV-specific CD<sub>8</sub> T cells can down-regulate T<sub>H</sub>1 cell type 2 cytokines and eosinophilia during RSV infection (35), and the regulation of these cytokine responses has been shown to be mediated by IFN-γ (36). RSV-specific CTL induced by DNA vaccines may play a regulatory role by contributing T<sub>H</sub>1 CD4<sup>+</sup> lymphocyte differentiation. These data raise the possibility that epitope-based DNA immunization may be an effective vaccine strategy to selectively activate protective T cell subsets in an optimal microenvironment, whilst avoiding unwanted or potentially deleterious responses.

Acknowledgements
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CTL responses induced by epitope-based DNA vaccines

Abbreviations

CMV cytomegalovirus
CTL cytotoxic T lymphocyte
ER endoplasmic reticulum
i.c. intracranial
i.d. intradermal
i.n. intranasal
MV measles virus
RSV respiratory syncytial virus

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