Enhancement of DNA vaccine potency through linkage of antigen to filamentous bacteriophage coat protein III domain I

Ángel M. Cuesta,[1,*] Eduardo Suárez,[1,*] Martin Larsen,[2] Kim Bak Jensen,[2] Laura Sanz,[1] Marta Compte,[1] Peter Kristensen[2] and Luis Álvarez-Vallina[1]

[1]*Servicio de Inmunología, Hospital Universitario Puerta de Hierro, Madrid, Spain, and* [2]*Department of Molecular Biology, University of Aarhus, Aarhus, Denmark*

doi:10.1111/j.1365-2567.2006.02325.x
Received 9 June 2005; revised 18 November 2005; accepted 25 November 2005.
*Ángel. M. Cuesta and Eduardo Suárez contributed equally to this work.
Correspondence: Dr Luis Álvarez-Vallina, Servicio de Inmunología, Hospital Universitario Clínica Puerta de Hierro, San Martín de Porres 4, 28035 Madrid, Spain.
Email: lalvarezv.hpth@salud.madrid.org
Senior author: Dr Luis Álvarez-Vallina, email: lalvarezv.hpth@salud.madrid.org

Summary

Although DNA-based cancer vaccines have been successfully tested in mouse models, a major drawback of cancer vaccination still remains, namely that tumour antigens are weak and fail to generate a vigorous immune response in tumour-bearing patients. Genetic technology offers strategies for promoting immune pathways by adding immune-activating genes to the tumour antigen sequence. In this work, we converted a model non-immunogenic antigen into a vaccine by fusing it to domain I of the filamentous bacteriophage coat protein III gene. Vaccination with a DNA construct encoding the domain I fusion generated antigen-specific T helper 1-type cellular immune responses. These results demonstrate that the incorporation of protein III into a DNA vaccine formulation can modulate the gene-mediated immune response and may thus provide a strategy for improving its therapeutic effect.

Keywords: DNA vaccine; genetic fusion; bacteriophage

Introduction

Improvement of vaccine efficacy has become a key goal in the development of DNA vaccination as an antitumour therapy. A prerequisite for successful cancer vaccination is breaking the tolerance to tumour-associated antigens, which represent 'self-antigens' and are therefore poorly immunogenic. Various DNA–fusion vaccine strategies, whereby antigens are genetically linked to immuno-enhancing molecules, have been explored.[1] These immuno-activating genes include cytokines,[2] chemokines,[3] costimulatory molecules[4] and pathogen-derived sequences.[5–7]

Bacterial virus particles, such as filamentous bacteriophage, have been found to exhibit an adjuvant-like effect when applied in vaccination strategies.[8] Furthermore, it has been shown that the immune response to single-chain antibody fragments (scFvs) is significantly improved by the fusion or co-administration of fragments of coat protein III of filamentous bacteriophage (M. Larsen *et al.*, submitted). The adjuvant and carrier properties of the functionally fused antibody (FuncFAb) system[9] may be the result of multimerization of the scFv fusions to coat protein III, leading also to potential heteromultimerization (ref. 9 and M. Larsen *et al.*, submitted).

Here, we show that the domain I fragment of coat protein III of the filamentous bacteriophage (DI) can induce immunity when delivered as a fusion gene with an scFv. The DNA immunization efficiently primed the immune system, as indicated by the strong T helper 1 (Th1) humoral and cell-mediated antigen-specific immune response, elicited by the expressed DI fusion protein.

Abbreviations: BPBS, 3% BSA in PBS; BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; DI, domain I fragment of filamentous bacteriophage coat protein III; DMEM, Dulbecco's modified Eagle's minimal essential medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FuncFAb, functionally fused antibody; IFA, incomplete Freund's adjuvant; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; IPTG, isopropyl-β-D-thiogalactopyranoside; i.p., intraperitoneal; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; POD, peroxidase; scFv, single-chain antibody fragment; Th1, T helper 1.

Materials and methods

Construction of vectors

The DI fragment was amplified from plasmid pKBJ3[9] with primer pair pKBJ3NotI (5′-AGGTGCGGCCGCCG GGGCCGCAACTGTTGA-3′) and pKBJ3XbaI (5′-AAA ATCTAGACCAGTGAATTCTTATTAATG-3′). The *Not*I/*Xba*I-cleaved polymerase chain reaction (PCR) fragment was ligated into the *Not*I/*Xba*I-digested backbone of plasmid p.L36,[10] resulting in p.L36-DI. The sequence was verified using primer BGHreverse (5′-TAGAAGGCACAG TCGAGG-3′). Plasmid DNA was purified using the Endofree plasmid megakit (Qiagen, Hilden, Germany).

Expression and purification of recombinant antibodies

L36 is an antilaminin scFv fragment that inhibits angiogenesis both *in vitro* and *in vivo*, and was selected from a human phage display library.[10] The expression and purification of non-fused and DI-fused L36 antibodies were performed as described previously.[9] Phage particles from selected clones were used to infect *Escherichia coli* HB2151 cells. Single colonies were grown overnight at 37° in 2 × TY medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl per liter) supplemented with 100 μg/ml ampicillin and 1% glucose. Five hundred millilitres of 2 × TY medium containing 100 μg/ml ampicillin and 0·1% glucose was inoculated into 5 ml of the overnight culture and incubated for 4 hr at 37° with shaking. The cultures were induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown overnight at 28°. Periplasmic fractions were prepared by osmotic shock and cleared by centrifugation prior to immobilized metal affinity chromatography (Amersham Biosciences, Uppsala, Sweden). The purified antibodies were verified by western blotting and enzyme-linked immunosorbent assay (ELISA) using the anti-myc 9E10 monoclonal antibody (mAb). Briefly, Maxisorp® 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4° with laminin-1 (Sigma Biosciences, St Louis, MO) at a concentration of 1 μg/well. After three washes, plates were blocked for 2 hr at room temperature with 4% non-fat dry milk in phosphate-buffered saline (PBS), then antibody derivatives were added (at varying concentrations) for 1 hr at room temperature. After three washes, bound antibody was detected using the 9E10 mAb.

Culture conditions and cell transfections

Human embryonic kidney 293T cells (ATCC CRL-1573) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen Life Technologies, Carlsbad, CA). 293T cells were transfected with plasmids pCR3.1, p.L36 or p.L36-DI using Lipofectamine (Invitrogen Life Technologies). Supernatants were collected 48 hr post-transfection, and the secretion of functional scFv was demonstrated by ELISA[10] and western blot, as described above.

Animals and immunizations

Two groups of BALB/c mice (6–8-week-old females; Harlan Ibérica, Barcelona, Spain) were immunized with PBS ($n = 4$) or recombinant L36 (50 μg) emulsified in 100 μl of incomplete Freund's adjuvant (IFA) ($n = 4$). Injections were given intraperitoneally (i.p.) on days 0 and 14. Two groups of mice ($n = 4$) were injected with 50 μg of plasmid DNA (pCR3.1–L36 or pCR3.1–L36-DI). Mice received bilateral injections (25 μg each) into left and right posterior thigh muscles on days 0, 7, 14 and 21.[11] Mice were bled by retro-orbital puncture at regular time-points and plasma was stored at −20° until assayed. All animals were handled in accordance with the guidelines of the Department of Health and Human Services.

Evaluation of immune response

L36-specific antibodies were detected by ELISA, using Maxisorp® 96-well plates coated overnight at 4° with recombinant scFv (L36 or B1.8) or bovine serum albumin (BSA) (0·25 μg/well). After three washes, plates were blocked for 30 min at room temperature with 3% BSA in PBS (BPBS), then diluted sera in 3% BPBS was added and the plates were incubated for 1 hr at room temperature. After three washes, immunoglobulin G (IgG)1 and IgG2a isotypes and total IgG were detected by incubation with anti-mouse IgG1, IgG2a and IgG polyclonal rabbit antibodies (Abcam, Cambridge, UK), respectively. After additional washing, a peroxidase (POD)-conjugated goat anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) was used for detection.

Antigen-specific cytokine assays

Red blood cell-depleted splenocytes (2×10^5) were plated in 100 μl of RPMI supplemented with 10% FCS in round-bottom 96-well microtitre plates (Corning Inc., Corning, NY). The splenocytes were stimulated with purified bacterial-produced L36 scFv (7·5 μg/ml), or with medium alone as a control for the baseline level of cytokine synthesis. After 72 hr, supernatants were harvested and assayed for interleukin-4 (IL-4) and interferon-γ (IFN-γ) using commercially available ELISA kits (Endogen, Woburn, MA). Antigen-specific cytokine secretion was obtained by subtracting the cytokine content of splenocytes incubated with complete medium alone.

Serum cytokine determination

Serum cytokine levels were determined using the cytometric bead array kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. Cytometric analysis was performed by FACScalibur (Becton Dickinson, Bedford, MA) and data analysis was achieved using the software BMS-FFS (Bender MedSystems).

Results

Construction of a DI-fused L36-encoding mammalian expression vector

We have previously described and characterized the human cytomegalovirus (hCMV)-driven mammalian expression vector, p.L36, containing the antilaminin-1 L36 scFv immunoglobulin.[10] In order to study the ability of mammalian cells to secrete a filamentous phage gene III DI-fused scFv immunoglobulin, we constructed the plasmid p.L36-DI, containing the human oncostatin M leader sequence followed by L36 scFv fused to the first 80 translated non-leader sequence codons of gene III (Fig. 1a). Transfection of human 293T cells with plasmid p.L36 or p.L36-DI resulted in the secretion of functional antibodies. The secreted non-fused and DI-fused scFvs bound specifically to their cognate antigen (laminin-1), as determined by ELISA (Fig. 1b). Antibody was not detected in the cell culture supernatant of 293T cells transfected with pCR3.1 control plasmid (Fig. 1b,c). Western blot analysis of conditioned medium from 293T cells transfected with p.L36 or p.L36-DI plasmids demonstrated that the migration pattern of the secreted proteins was consistent with the predicted molecular weight (Fig. 1c). Under reducing conditions, the migration patterns of both mammalian- and bacterial-produced non-fused L36 antibodies were similar, whereas the mammalian-produced DI-fused L36 scFv was slightly larger than the bacterial-produced DI fusion, probably owing to differences in the glycosylation pattern. In fact, DI has a predicted *N*-glycosylated site (residues 57–60).[12]

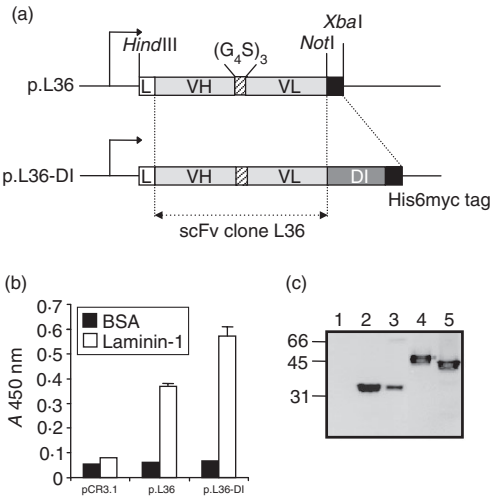


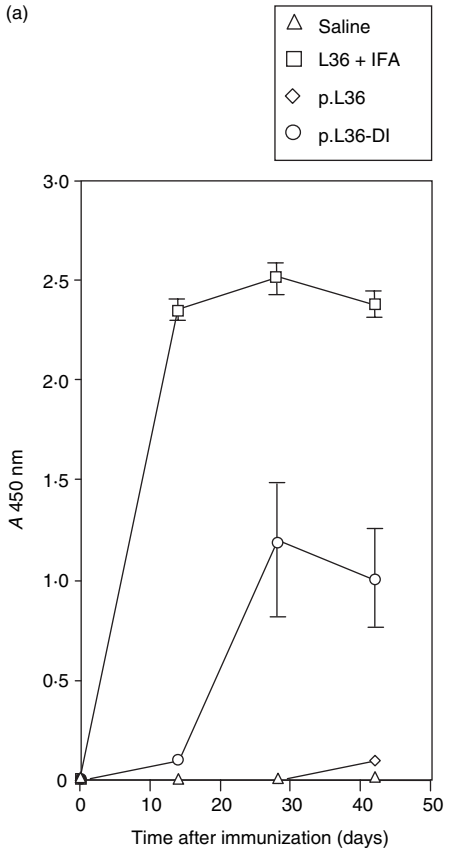
Figure 1. (a) Schematic representation of single-chain antibody fragment (scFv) gene constructs. The direction of transcription is indicated by arrows. The His6-myc tag was appended for immunodetection. DI, domain I; L, oncostatin M leader sequence; VH, V-domain of the Ig heavy chain; VL, V-domain of the Ig light chain. (b) Secretion of functional L36 scFv into the cell culture supernatant by human 293T cells transfected with plasmid pCR3.1, plasmid p.L36 or plasmid p.L36-DI. The functionality of scFv antibodies was demonstrated by enzyme-linked immunosorbent assay (ELISA) against plastic-immobilized human bovine serum albumin (BSA) and laminin-1. (c) Western blot analysis of scFv antibodies secreted into the cell culture supernatant by 293T cells transfected with plasmid pCR3.1 (lane 1), plasmid p.L36 (lane 2) or plasmid p.L36-DI (lane 4). Lane 3, recombinant L36 non-fused to DI purified from bacteria, and lane 5, recombinant L36 fused to DI purified from bacteria.

Vaccination and immune response

Mice were immunized with plasmids encoding non-fused and DI-fused L36 scFvs, and their antigen-specific humoral responses were compared with those of the mice injected with purified non-fused L36 scFv mixed with IFA. Anti-L36 IgG was detected in mice immunized with purified non-fused scFv on day 14, and with plasmid p.L36-DI on day 28, after the third DNA injection (Fig. 2a,b). The anti-L36 IgG induced by both the purified non-fused L36 scFv and the plasmid DNA encoding the DI-fused L36 showed no reactivity by ELISA with an unrelated scFv derived from the hapten-specific mAb B1.8 (data not shown). Immunization with plasmid containing only the scFv sequence failed to induce detectable anti-L36 scFv immunoglobulin (Fig. 2a). In mice immunized with p.L36-DI, the subclass distribution of IgG1 and IgG2a showed dominance of the IgG2a isotype (Fig. 3a) characteristic of a Th1-type response. In mice immunized with purified L36 scFv mixed with IFA, the subclass distribution of IgG1 and IgG2a showed higher IgG1 than IgG2a levels, although both subclasses were prominent (Fig. 3a). A similar subclass distribution of IgG1 and IgG2a was observed in mice immunized with purified L36 scFv alone, or fused to the DI of protein III (M. Larsen *et al.*, submitted).

Antigen-specific cytokine production

The cellular response induced in mice by immunization with p.L36-DI was also measured. For that purpose, we examined the cytokine production profile of antigen-specific immune cells. Splenic mononuclear cells were obtained 1 week after the last plasmid DNA immunization (day 28) and were stimulated *in vitro* with the purified non-fused L36 scFv. After 72 hr, the supernatants were harvested and assayed for both IL-4 and IFN-γ. The results, depicted in Fig. 3, indicated that immunization with p.L36-DI stimulates the production of large quantities of IFN-γ (Fig. 3b),



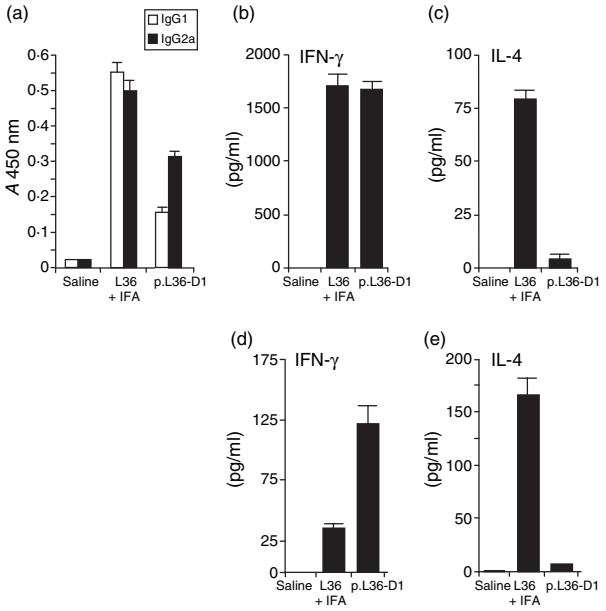


Figure 3. Isotype response and cytokine production in BALB/c mice following immunization with recombinant non-fused L36 single-chain antibody fragment (scFv) + IFA or p.L36-DI plasmid DNA. (a) The sera were harvested on day 42, diluted (the dilution was 1 : 1000 in the L36 + IFA group and 1 : 25 in p.L36-DI plasmid group) and tested for laminin-specific IgG1 and IgG2a responses by enzyme-linked immunosorbent assay (ELISA). Results are the mean value ± standard error (SE) of four mice per group. (b, c) Cytokine ELISA of supernatants from splenocytes of different groups restimulated *in vitro* with purified non-fused L36 scFv (7·5 μg/ml). The cell-free supernatants were tested for interferon-γ (IFN-γ) (b) and interleukin-4 (IL-4) (c). (d, e) The sera were assayed, as described in the Materials and methods, to determine the cytokine profile.

but almost undetectable levels of IL-4 (Fig. 3c). In contrast, high levels of IL-4 were observed in the supernatants of splenocytes obtained from animals immunized with purified non-fused L36 scFv mixed with IFA (Fig. 3c). In these supernatants, IFN-γ was detected at considerable levels.

Cytokine levels in the serum of immunized mice

Sera obtained when the mice were killed were also assayed for cytokine content by using a cytometric bead array kit. As

Figure 2. (a) Antigen-specific antibody responses in BALB/c mice following immunization with recombinant non-fused L36 single-chain antibody fragment (scFv) + incomplete Freund's adjuvant (IFA) (50 μg on days 0 and 14) or DNA vaccines (50 μg of p.L36 or p.L36-DI on days 0, 7, 14 and 21). The antigen-specific immunoglogulin G (IgG) response (serum dilution 1 : 25) was determined by enzyme-linked immunosorbent assay (ELISA) on days 14, 28 and 42. Each symbol represents the mean value ± standard error (SE) of four mice. (b) Titration of IgG responses following immunization with recombinant non-fused L36 scFv + IFA or p.L36-DI plasmid DNA. The pooled sera ($n = 4$) were tested for laminin-specific IgG responses on day 42.

seen in Fig. 3d, treatment with p.L36-DI resulted in significantly elevated levels of IFN-γ. IL-4 (Fig. 3e) was almost undetectable in mice immunized with p.L36-DI, in contrast to the animals immunized with purified non-fused L36 scFv.

Discussion

We have demonstrated the feasibility of using the N-terminal domain of filamentous bacteriophage coat protein III as an adjuvant molecule in a genetic vaccination protocol. We converted a model, non-immunogenic scFv fragment into a vaccine by fusing it to the first 80 residues of protein III.

With the appropriate immunization schedule, a naked DNA plasmid vaccine encoding the scFv–DI fusion induced a Th1 immune response, characterized by a dominant L36-specific IgG2a humoral response and high levels of IFN-γ produced by splenic T cells. Immunity was not elicited by naked DNA plasmid encoding non-fused sFv. Immunization with purified non-fused scFv protein and adjuvant elicited a mixed Th1/Th2 cellular immune response. Th1 immunity has classically been considered to be more attractive in a therapeutic setting, given that the associated high cytotoxic T-lymphocyte (CTL) activity plays a key role in the host defence against tumours and viral infections.

The differences observed in the present study could be explained by the fact that plasmid DNA uptake results in an endogenous synthesis of the encoded protein and its subsequent association with major histocompatibility complex (MHC) class I molecules; in contrast, administered soluble proteins are processed predominantly through the exogenous pathway. Activation of the immune response is also known to be influenced by both the nature and molecular form of the antigen, with large aggregates being particularly immunogenic.[13] Previous results suggest that aggregated antigen amplifies a Th1-type response, already activated by the cytokine environment generated by the injection of DNA.[14] Induction of Th1-type dominant responses has also been noted using genetic fusions of aggregating plant viral coat proteins to both peptides and scFvs.[7,15,16] In our model, multimerization of the scFv is accomplished by linking the antibody fragment to domain I of coat protein III when examined *in vitro*.[9] This fusion design has the added advantage of functioning as a 'built-in' adjuvant, creating a direct route from reactive generation to vaccine development.

Acknowledgements

This work was supported by grants from the Fondo de Investigaciones Sanitarias (02/1144), the CAM (GR/SAL/0214/2004) and the 5th framework of the European Community (grant ECQLK3-CT-1999-00386) (to L.Á-V), and by grants from the Danish Technical Research Council and the Novo Nordisk Foundation (to P.K). L.S. is an investigator from the Ramon y Cajal Program (Ministerio de Educación y Ciencia). M.L and K.B.J. have been supported, in part, by NEMOD Immunotherpies AG.

References

1 Zhu D, Stevenson FK. DNA gene fusion vaccines against cancer. *Curr Opin Mol Ther* 2002; **4**:41–8.
2 Syrengelas AD, Chen TT, Levy R. DNA immunization induces protective immunity against B-cell lymphoma. *Nat Med* 1996; **2**:1038–41.
3 Biragyn A, Tani K, Grimm MC, Weeks S, Kwak LW. Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. *Nat Biotechnol* 1999; **17**:253–8.
4 Conry RM, Widera G, LoBuglio AF *et al.* Selected strategies to augment polynucleotide immunization. *Gene Ther* 1996; **3**:67–74.
5 Spellerberg MB, Zhu D, Thompsett A, King CA, Hamblin TJ, Stevenson FK. DNA vaccines against lymphoma: promotion of anti-idiotypic antibody responses induced by single chain Fv genes by fusion to tetanus toxin fragment C. *J Immunol* 1997; **159**:1885–92.
6 Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM, Wu TC. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res* 2000; **60**:1035–42.
7 Savelyeva N, Munday R, Spellerberg MB, Lomonossoff GP, Stevenson FK. Plant viral genes in DNA idiotypic vaccines activate linked CD4+ T-cell mediated immunity against B-cell malignancies. *Nat Biotechnol* 2001; **19**:760–4.
8 Willis AE, Perham RN, Wraith D. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene* 1993; **128**:79–83.
9 Jensen KB, Larsen M, Pedersen JS, Christensen PA, Álvarez-Vallina L, Goletz S, Clark BF, Kristensen P. Functional improvement of antibody fragments using a novel phage coat protein III fusion system. *Biochem Biophys Res Commun* 2002; **298**:566–73.
10 Sanz L, Kristensen P, Blanco B, Facteau S, Russell SJ, Winter G, Álvarez-Vallina L. Single-chain antibody-based gene therapy: inhibition of tumor growth by *in situ* production of phage-derived human antibody fragments blocking functionally active sites of cell-associated matrices. *Gene Ther* 2002; **9**:1049–53.
11 Stevenson FK, Zhu D, King CA, Ashworth LJ, Kumar S, Hawkins RE. Idiotypic DNA vaccines against B-cell lymphoma. *Immunol Rev* 1995; **145**:211–28.
12 Hill DF, Petersen GB. Nucleotide sequence of bacteriophage f1 DNA. *J Virol* 1982; **44**:32–46.
13 Jagadish MN, Edwards SJ, Hayden MB *et al.* Chimeric potyvirus-like particles as vaccine carriers. *Intervirology* 1996; **39**:85–92.
14 Roman M, Martin-Orozco E, Goodman JS *et al.* Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med* 1997; **3**:849–54.
15 King CA, Spellerberg MB, Zhu D *et al.* DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat Med* 1998; **4**:1281–6.
16 McInerney TL, Brennan FR, Jones TD, Dimmock NJ. Analysis of the ability of five adjuvants to enhance immune responses to a chimeric plant virus displaying an HIV-1 peptide. *Vaccine* 1999; **17**:1359–68.

