

Enhancement of Antibody Responses in DNA Vaccination Using a Vector Encoding a Universal T-Helper Cell Epitope

SYLVIE TYMCIU,¹ CORALIE DURIEUX-ALEXANDRENNE,¹ ANNE WIJKHUISEN,^{1,2}
CHRISTOPHE CRÉMINON,¹ YVELINE FROBERT,¹ JACQUES GRASSI,¹
JEAN-YVES COURAUD,^{1,2} and DIDIER BOQUET¹

ABSTRACT

DNA vaccination appears as a very promising approach to raise protective antibodies against a variety of proteins from pathogens or tumor cells, but is often hindered by the low immunogenicity of the genetic vectors used for the immunizations. To enhance the humoral response through improvement of the antigenic presentation of newly synthesized proteins upon vaccination, we engineered a plasmid coding for a low immunogenic protein (an scFv, i.e. the single-chain Fragment variable of a well-characterized antibody) fused to a small-size universal T-helper cell epitope derived from tetanus toxin, whose efficiency in classical protein-based immunization protocols has already been demonstrated. We found that immunization of C57Bl/6 mice using this vector greatly enhanced the production not only of specific antibodies recognizing essentially conformational epitopes on the undenatured scFv protein but also of antibodies against linear epitopes on the denatured protein. Since this T-epitope is known to be accommodated by several haplotypes of H-2 molecules in mice, as well as by various class II MHC molecules in humans, the results reported here allow us to conclude that this method could be of general interest for future applications of genetic immunization, including DNA-based vaccinations in humans.

INTRODUCTION

IN RECENT YEARS, DNA vaccination has been found to be a promising alternative strategy to classical protein or peptide immunizations for inducing protective immunity against a range of pathogens (Hasan *et al.*, 1999; Sullivan *et al.*, 2000; Epstein *et al.*, 2002; Muthumani *et al.*, 2003) or for the treatment of cancers (Zhu *et al.*, 1997; Kim *et al.*, 2001) and allergies (Raz *et al.*, 1996). One problem generally encountered, however, was the relatively low immunogenicity of plasmid DNA vaccines compared to their protein counterparts (Zhu *et al.*, 2001). Different strategies have thus been developed to enhance this immunogenicity: a classical one consists of incorporating immune stimulatory sequences (e.g., unmethylated CpG dinucleotides) into the immunogen plasmid to facilitate its endocytosis by antigen-presenting cells through Toll-like receptors (Medzhitov, 2001; Chuang *et al.*, 2002; Kaisho and Akira, 2002), which also stimulates the production of interleukines and interferon (Klin-

man *et al.*, 1996; Warren *et al.*, 2000). Another strategy which proved its efficacy is to use plasmids containing additional sequences coding for a variety of molecules with different immunological properties or functions, for example, cytokines (IL2, IL12, IL15) (Sin *et al.*, 2000; Boyer *et al.*, 2002), chemokines (GM-CSF) (review by Cohen *et al.*, 1998), co-stimulatory molecules (B7-1, B7-2, or CTLA4) (Conry *et al.*, 1996; Corr *et al.*, 1997; Iwasaki *et al.*, 1997; Kim *et al.*, 1997; Mendoza *et al.*, 1997; Agadjanyan *et al.*, 2003) or ubiquitin (Rodriguez *et al.*, 1997; Wu and Kipps, 1997), these proteins being either coexpressed with the antigen of interest or, alternatively, expressed as fusion proteins. Finally, a conceptually different approach to the optimization of DNA vaccines is to maximize the generation of T-cell epitopes from encoded gene products, which improves the presentation of antigen-derived peptides through MHC class I and class II molecules and, as a consequence, enhances both cellular and humoral responses, respectively. For this purpose, DNA fusion vaccines have been

¹CEA, Service de Pharmacologie et d'Immunologie, DSV/DRM, Bât. 136, CEA/Saclay, 91191 Gif-sur-Yvette Cedex, France.

²Université Paris 7-Denis Diderot, UFR de Biologie, 75251 Paris Cedex, France.

constructed with various proteins already known to behave as efficient carriers in conventional peptide and protein-based vaccines (Benvenuti *et al.*, 2000; Armstrong *et al.*, 2002). In this respect, DNA vaccines composed of antigens genetically linked to the nontoxic C fragment (FrC) of tetanus toxin (TT), have been shown to induce strong protective immunity (Spellerberg *et al.*, 1997; King *et al.*, 1998; Rice *et al.*, 2001; Forconi *et al.*, 2002). Trying to enhance the efficiency of conventional protein-based immunizations, Kumar *et al.* (1992) reported that TT or FrC (which corresponds to the 50-kDa carboxyl-terminal portion of the heavy chain of TT), could be favorably replaced by a short 15-mer peptide, a so-called universal T-helper cell epitope, located in the first domain of FrC (aa 829–844). The use of this small-size peptide as a carrier, instead of FrC or TT, was also particularly interesting in that it allowed to overcome the problem of epitope-specific suppression, in which induction of an immune response against a synthetic peptide conjugated to TT is prevented by preexisting immunity to TT, as would be the case for a large part of the human population. In this context, the aim of the present work was to test the immunogenicity of a genetic vaccine designed by fusing the DNA sequence coding for this well-defined small-size T-epitope to the gene of a protein of interest.

We chose a single-chain fragment variable (scFv) of an antibody as antigen in this study for two reasons: the first reason is that scFv have long been known to be poorly immunogenic proteins, and FrC from tetanus toxin has already been used successfully to enhance their immunogenicity in both protein-based and genetic immunizations (Spellerberg *et al.*, 1997). The second reason is that a major question to be addressed, especially when an antibody response is expected whatever the immunization procedures, is the structural conformation of the immunogen, on which will depend the ability of generated antibodies to recognize the antigen either in a native or a denatured form: in this respect, antibodies or their fragments used as immunogens offer a unique opportunity for testing a native conformation (at least of the paratope region) by performing antigen-antibody binding studies. In the present study, the cDNA coding for the scFv of an antibody (mAb SP31) directed to the neuropeptide substance P (SP), which we have extensively characterized previously (Couraud *et al.*, 1987; Déry *et al.*, 1996; Hanin *et al.*, 1997), was thus fused with the DNA sequence coding for the “universal” T-helper cell epitope of FrC defined above. First, we show that cultured cells, transfected *in vitro* with this construction (ScFv-T), were able to synthesize and secrete large amounts of the fusion protein, a significant fraction of which is expressed in a functional conformation. Above all, our results indicate that this T-epitope fusion procedure largely enhanced the antibody response against both undenatured and denatured forms of the scFv protein after DNA immunization of C57Bl/6 mice.

MATERIALS AND METHODS

Plasmid constructions

Production and binding properties of the mouse monoclonal antineurotensin P antibody (SP 31), as well as the cloning and construction of its scFv fragment have been already described in detail (Couraud *et al.*, 1987; Boquet *et al.*, 2000).

The cDNA of the scFv was inserted, at the SfiI/Not I sites, into the secretion vector pSecTag2a (Invitrogen, Carlsbad, CA) which has the same expression characteristics than pcDNA 3.1 vector (Invitrogen), our negative control for immunization experiments. Both vectors contain a cytomegalovirus promoter for a high-level constitutive expression and a bovine growth hormone polyadenylation signal and transcription termination sequence to enhance mRNA stability. The pSecTag2a contains an additional sequence encoding for a secretion signal from the V-J2-C region of the mouse Ig kappa chain allowing efficient secretion of recombinant proteins.

Construction of the scFv-T plasmid was realized by inserting the sequence coding for a universal T-helper epitope [corresponding to aa 829–844 of fragment C (FrC) from tetanus toxin] at EcoRI/HindIII sites of the ScFv plasmid through ligation of double-strand oligonucleotides with compatible restriction site extremities. Sequences of the two phosphorylated oligonucleotides were: AATTCC ATC CAG TAC ATC AAG GCT AAC TCC AAA TTC ATC GGT ATC ACC GAA CTG A and AGCCTT CAG TTC GGT GAT ACC GAT GAA TTT GGA GTT AGC CTT GAT GTA CTG GAT GG.

Transfection experiments and *in vitro* eukaryotic expression of scFv and scFv-T proteins

CHO cells were cultured in Ham F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin, at 37°C in a 5% CO₂ humidified atmosphere. The constructs harboring the scFv, scFv-T cDNA, or control empty vectors were transfected into CHO cells by using Lipofectamine Plus Reagent (Invitrogen). Three days after transfection, the culture supernatant was collected, and cytoplasmic soluble and insoluble extracts were prepared as follows: cells were discarded with 5 ml trypsin-EDTA and centrifuged at 1000 × g for 10 min at 4°C. The pellet was resuspended in 1.2 ml phosphate buffer 20 mM, pH 7.4 (PBS), sonicated for 15 sec, and centrifuged again at 12,000 × g for 10 min at 4°C. The supernatant was then referred to as the soluble cytoplasmic extract, while the pellet, which was resuspended in 0.12 ml phosphate buffer 20 mM, pH 7.4, corresponds to the insoluble cytoplasmic extract. Protein concentrations were measured by using the bicinchoninic acid method (Pierce, Rockford, IL); for each fraction (i.e., supernatant and both cytoplasmic extracts), the total protein respective concentrations were identical for cells transfected with scFv and scFv-T encoding vectors.

For a qualitative comparison of the levels of both total and functional scFv and scFv-T recombinant proteins in cell supernatants and cytoplasmic extracts, we used two different immunometric assays, as we recently described (Boquet *et al.*, 2000). Briefly, 100 µl of different samples (containing 50, 5, and 0.5 µg of proteins for supernatant, soluble, and insoluble cytoplasmic extracts, respectively) were dropped on microtiter plates coated with an anti-Ha-Tag monoclonal antibody (MAb 12CA5, Boehringer Mannheim, Indianapolis, IN, 10 µg/well). After 18 h at 4°C and washing, 100 µl of enzymatic tracer were added, which corresponds either to an anti-His-Tag antibody conjugated with acetylcholinesterase (AChE: EC 3.1.1.7, 2 Ellman U/ml) or to SP labeled with AChE (5 Ellman U/ml). After a further 18 h-incubation and washing, solid phase-bound

AChE activity was measured by addition of 100 μ l of Ellman medium (Pradelles *et al.*, 1985) and OD was read after 1-h reaction time. As discussed recently (Boquet *et al.*, 2000), these two ELISA tests allow the parallel detection of the entire recombinant scFv (regardless of its conformation) and of the active scFv (able to recognize its specific antigen SP, that is, presumably correctly folded), respectively.

Western blot experiments were also done on supernatants, soluble, and insoluble cytoplasmic extracts from transfected cells. Fifty micrograms of proteins from each sample were resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific protein binding was blocked with 5% dried milk in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% Tween 20. The nylon membranes were reacted overnight with a mouse MAb to Ha-Tag (12CA5) and, after washing, were reacted for 3 h with peroxidase-conjugated goat antimouse IgG antibody (1/10⁵) (Pierce). Staining was achieved using a chemiluminescent substrate (Pierce) and exposure of X-OMAT film (Eastman Kodak Company, Rochester, NY) according to the supplier's instructions.

Immunization schedules

Five 6-week female C57Bl/6 (H-2b) mice were immunized with the scFv cDNA vector (mice nos. 6–10), five mice were immunized with the scFv-T cDNA vector (mice nos. 11–15), while five mice (nos. 1–5), immunized with the empty vector, served as negative controls. All animals were immunized through intramuscular injections in the anterior tibialis muscle on day 0 with 50 μ g of plasmid in PBS, 5 days after pretreatment of the muscle with cardiotoxin purified from venom of *Naja nigricollis* (10 μ M, 50 μ l/injection) (Latoxan, Valence, France). Injections were repeated at weeks 3, 6, and 15 thereafter. Blood samples were obtained from retroocular capillaries at week 0 (preimmune bleeding) and at weeks 2, 5, 9, 12, and 17 after the initial injection.

Detection and analysis of specific anti-scFv antibodies by ELISA, binding experiments, and Western blot

Detection of anti-scFv antibodies in successive immune sera, recognizing a native form of scFv proteins, was carried out through immunometric assays, using microtiter plates coated with undenatured scFv immobilized through rabbit polyclonal antibodies directed against the His-Tag. Assays were performed using a sequential procedure: in the first capture step, 100 μ l of the cytoplasmic soluble extract from CHO cells transfected with the scFv encoding vector (prepared as described above and containing scFv in a functional conformation, see Results) were reacted for 18 h at 4°C. After washing, serum samples (diluted 1/1000 in EIA buffer, 100 μ l/well) were added overnight at 4°C. Then, plates were washed three times, and 100 μ l of antimouse IgG goat antibodies labeled with AChE (2 Ellman U/ml) were reacted overnight at 4°C. After washing, solid phase-bound AChE activity was measured by addition of 200 μ l of Ellman medium and OD was read after 1-h reaction time.

Competition binding experiments were then performed to investigate whether any anti-scFv antibodies could be directed against the paratope of the scFv protein, that is, the binding site of the antigen SP. For that purpose, increasing concentrations of the competitor SP (up to 100 μ g/ml) were added to serum

samples during the overnight incubation at 4°C in the preceding immunometric assay. All other steps were identical.

Finally, detection of anti-scFv antibodies in successive immune sera, recognizing a denatured form of scFv proteins, was carried out through Western blot experiments, using periplasmic extracts from bacteria expressing high levels of recombinant scFv molecules (as we recently reported: Essono *et al.*, 2003). Briefly, after electrophoretic separation, under denaturing conditions, of 200 μ l of extracts and subsequent blotting (using the multiscreen system apparatus of Biorad), the nylon membranes were reacted overnight with sera from immunized mice (1/100). After washing, membranes were reacted for 3 h with peroxidase-conjugated goat antimouse IgG antibody (1/10⁵) (Pierce). Staining was achieved using a chemiluminescent substrate (Pierce).

RESULTS

In vitro expression of scFv or scFv-T

Preliminary experiments were performed to check the functionality of our constructs and especially to investigate the possible effects of the incorporation in the plasmid of the T-epitope coding sequence on (1) expression, (2) secretion, and (3) conformation of the scFv proteins in transfected cells. Assuming that the transfection level is similar for either vector, the data reported in Figure 1A indicated that the presence of the T-epitope did not grossly reduce the amount of secreted scFv proteins recovered in cell supernatants. This result is worth noting, since it suggests that an efficient secretion of newly synthesized epitope T-fused proteins could be reasonably expected *in vivo* after DNA immunization, a necessary condition to trigger a humoral immune response. By contrast, the amounts of cytoplasmic scFv proteins recovered in a soluble or an insoluble form were either largely (by fourfold) or slightly (by about 30%) enhanced by the addition of the T-epitope, possibly reflecting an increased synthesis of scFv-T compared to unfused scFv proteins, which could lead to enhanced aggregation. No signal was recovered in either extract when cells were transfected with the empty vector.

Immunoblotting experiments carried out on supernatants (inset Fig. 1A), soluble and insoluble cytoplasmic fractions (not shown) of cells transfected either with a scFv or scFv-T cDNA encoding plasmid, confirmed the presence of proteins at approximately 35 and 37 kDa, that is, the expected molecular weight of scFv and scFv-T, respectively.

Finally, the efficiency of the constructs in generating correctly folded scFv proteins was checked through functional assays, that is, by assessing the ability of newly synthesized and secreted scFv or scFv-T to recognize the SP peptide, the specific antigen for this antibody fragment. This test was neither applied to the cytoplasmic insoluble extracts (since proteins found in these samples were recovered using denaturing buffer which obviously strongly alters their native conformation) nor to fractions from control cells transfected with the empty vector. The results reported in Figure 1B indicated that the amount of functional scFv-T (as judged from the ability to bind the peptide target) was three to five times lower than the amount of functional unfused scFv in the soluble cytoplasmic extracts and

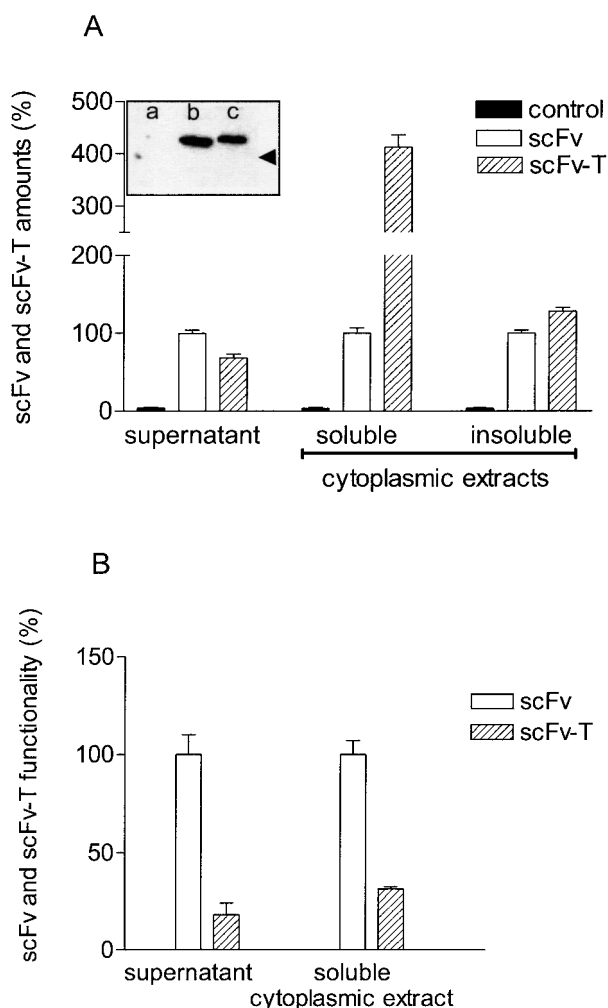


FIG. 1. Qualitative comparison of scFv protein expression (A) and functionality (B) in culture supernatant, cytoplasmic soluble, and insoluble extracts from CHO cells transfected *in vitro* with the vector coding for the scFv protein (empty bars), the vector coding for the scFv fused with the T epitope (scFv-T, hatched bars), or the control empty vector (filled bars). The scFv or scFv-T protein expression was measured through immunometric assay involving two antitag antibodies, while the functionality of the scFv or scFv-T was assessed from antigen-scFv (or scFv-T) binding experiments, as detailed in Materials and Methods. Results are expressed as percent of the signals (OD/h) measured, for each extract, using samples from CHO cells transfected with unfused scFv encoding plasmid (100%) and represent the mean $\pm$ S.D. of six determinations. Inset: Western blot of culture supernatants from CHO cells transfected with the control empty vector (a), the vector coding for the scFv (b), or the vector coding for the scFv-T protein (c) (arrow head: 30 kDa).

in the cell supernatants, suggesting that the addition of the T-epitope could significantly alter the conformation of the scFv paratope.

Since these *in vitro* preliminary experiments indicated together that both plasmids were actually able to trigger the synthesis and generate the secretion of large amounts of scFv or

scFv-T proteins, a fraction of them (which, however, remains difficult to estimate accurately) apparently being correctly folded, we then compared the respective immunogenicity of these DNA vectors after *in vivo* inoculation in mice.

Generation of antibodies against the undenatured form of the scFv

After protein or genetic immunizations, the presence of antibodies in successive immune sera is generally detected using ELISA tests involving the direct coating of the antigen on a solid phase. This procedure, however, not only requires large amounts of purified antigen in a native form (which, in the present case, we do not have at our disposal), but is also known generally to induce both structural modifications of the antigen and the masking of some epitopes (Butler *et al.*, 1992). For these reasons, we preferred to immobilize the antigen (i.e., scFv proteins) indirectly by using an antitag capture antibody. It is worth noting in addition that these immobilized scFv proteins (prepared from the cytoplasmic soluble extracts from scFv cDNA-transfected cells) were indeed in a native functional conformation (Fig. 1B): this means that this immunometric assay was considered here to detect preferentially, in the sera of animals immunized with either plasmid vector, anti-scFv antibodies specifically recognizing conformational epitopes of scFv molecules. As shown in Figure 2, our results clearly indicate that the scFv-T cDNA encoding vector was much more immunogenic than the plasmid coding for the unfused scFv. Indeed, whereas only one animal out of five immunized with the unfused scFv cDNA-containing plasmid (Fig. 2B) produced anti-scFv antibodies (which reached significant amounts only after 17 weeks and four DNA injections), considerably higher amounts of anti-scFv antibodies were recovered in most animals (three out of five) immunized with the scFv-T cDNA coding vector (Fig. 2C). In these animals, anti-scFv antibodies appeared at the second bleeding (i.e., after only 5 weeks and two DNA injections) and above all remained at elevated levels until at least the 17th week. The signals recovered using all sera from the five control mice immunized with the empty vector remained at the background level (Fig. 2A).

Since the recombinant scFv protein used to detect anti-scFv antibodies in the present ELISA test contained Ha, Myc, and His-tag sequences, the specificity of antibodies (both in all the preimmune sera and in pools of sera from bleedings two to five for all animals) was checked through two kinds of control assays, using either (1) plates coated with Ha, Myc, or His-tag and AChE-labeled antimouse IgG antibodies as the tracer, or (2) plates coated with antimouse IgG antibodies and AChE-labeled Ha, Myc, or His-tag as the peptide tracers. Neither format revealed the significant presence of antitag antibodies in either serum (not shown), indicating that antibodies are directed exclusively against scFv epitopes. Finally, neither serum from any mouse immunized with scFv or scFv-T cDNA encoding plasmid was found to inhibit significantly the binding of scFv to labeled-SP antigen, nor, conversely, was SP found to inhibit the binding of any antibody to immobilized scFv (not shown); these results indicate that anti-scFv antibodies produced by DNA-immunized animals were in their great majority either anti-idiotypic or Ab2 α anti-idiotypic antibodies, but do not, however, allow us to exclude the presence in the sera of small

amounts of (and/or low affinity) antiparatope Ab2 β antiidiotypic antibodies.

Generation of antibodies against the denatured form of the scFv

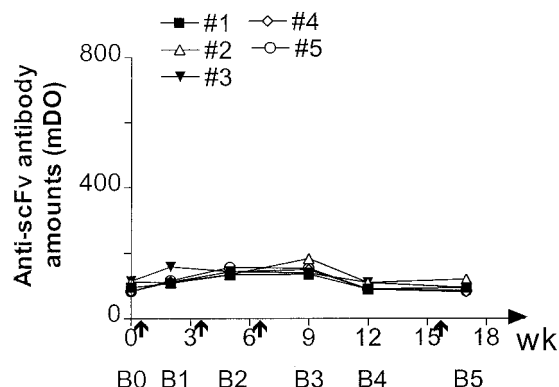
We also wondered in this study whether the generation of antibodies possibly recognizing linear epitopes of a denatured form of scFv was also enhanced by the presence of a T-epitope coding sequence in the immunizing plasmid. To this end, sera from all animals were systematically tested on Western blots with recombinant scFv proteins resolved by SDS-PAGE. As illustrated in Figure 3, some sera from mice immunized with the scFv-T cDNA-containing plasmid (but not their corresponding preimmune sera) did recognize a protein with the expected molecular weight of the recombinant scFv. By contrast, neither serum from any mouse immunized with the unfused scFv cDNA-containing plasmid nor from mice immunized with the control empty vector, gave a positive signal in this test (not shown). Data obtained with all sera from mice immunized with the scFv-T coding plasmid are qualitatively summarized in Table 1. It clearly appears that the use of a vector coding for a recombinant scFv fused to the T-epitope enhanced the humoral response against linear epitopes of the denatured protein even more greatly than the response against conformational epitopes of the undenatured protein: indeed, four animals out of five injected with scFv-T encoding plasmid generated antibodies to the denatured scFv, the peak response occurring 9–12 weeks after the first DNA injection (bleedings nos. 3–4). Interestingly, an inverse correlation seemed to occur between the antibody response against native and denatured forms of scFv protein, since the best-responding animals against conformational epitopes (mice nos. 13 and 14, Fig. 2C) produced no or very low levels of antidenatured scFv antibodies (Table 1), whereas the best-responding animals against denatured scFv were mice nos. 11 and 15 (Table 1), which generated no or low levels of antiundenatured scFv antibodies (Fig. 2C). Furthermore, when both kinds of antibodies were occasionally found to be produced by the same animal (mouse no. 14), they occurred with different kinetics, the antinative scFv antibodies appearing earlier than those against the antidenatured protein.

DISCUSSION

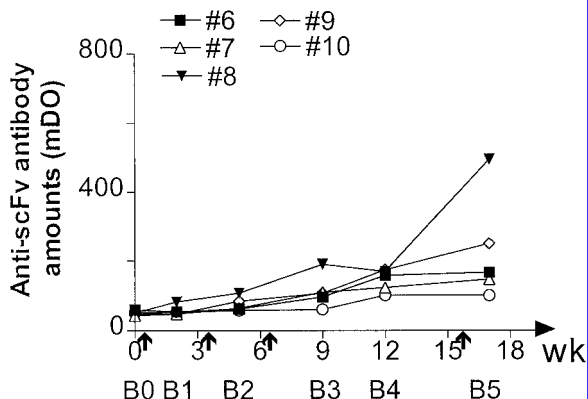
This study was primarily conducted to experiment with a new simple method of enhancing the immunogenicity of pro-

teins in DNA immunizations. Among the various approaches tested for that purpose until now, the fusion of pathogen-coding sequences (like the 50-kDa fragment C of tetanus toxin: FrC) to poorly immunogenic proteins has proven its efficiency (Spellerberg *et al.*, 1997; King *et al.*, 1998; Rice *et al.*, 2001; Forconi *et al.*, 2002). Here, we show that a more simple con-

A Control vector



B scFv



C scFv-T

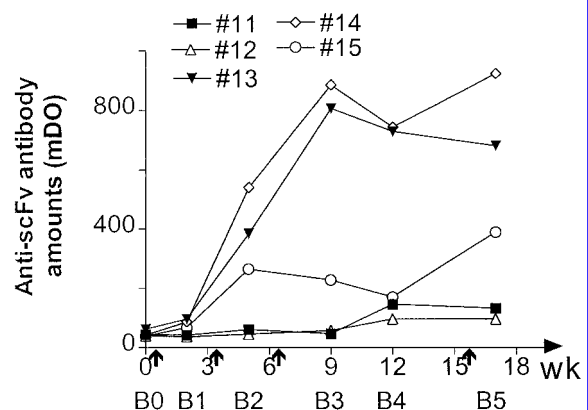


FIG. 2. Screening for antibodies recognizing the native scFv protein in the successive (preimmune: B0 and immune: B1 to B5) bleedings of mice immunized with the control empty vector (A), the vector coding for the scFv protein (B), or the vector coding for the scFv fused with the T epitope (scFv-T) (C). Arrows indicate plasmid injections. Immunometric assays were performed using undenatured scFv proteins retained on microtiter plates coated with antitag antibodies (see Materials and Methods). Results represent the mean of six determinations (SD, always lower than 10% of the corresponding absolute value, was not indicated for reason of clarity).

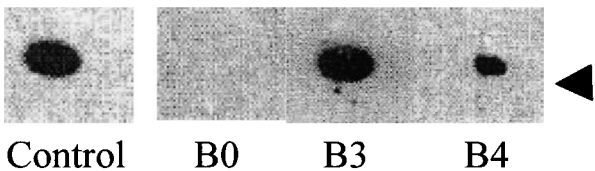


FIG. 3. Screening for antibodies recognizing the denatured form of scFv protein in selected sera (preimmune: B0, and immune bleedings: B3 and B4) of a mouse (no. 15) immunized with the vector coding for the scFv fused with the T epitope (scFv-T). Western blot experiments were performed using recombinant scFv proteins, submitted to gel electrophoresis under denaturing conditions (see Materials and Methods). After blotting, nylon membranes were either probed with peroxidase-labeled anti-Ha Tag antibody (Control) or reacted with the different sera before probing with peroxidase-conjugated goat anti-mouse IgG antibody (arrow head: 30 kDa).

struct, designed by fusing the cDNA of a single T-epitope (16 amino acids) of FrC to the sequence coding for a poorly immunogenic protein (the variable fragment of an antibody: scFv) largely enhanced the production of anti-scFv antibodies. Although it has been known for more than 10 years that this T-epitope improves the immunogenicity of synthetic peptides in classical protein-based immunizations (Kumar *et al.*, 1992), no attempt had been made until now to assess its possible adjuvant effect in DNA immunization. Since the commercial vector used in the present study has been modified here to accommodate cDNAs of various proteins inside a Sfi I/Not I cassette, a major interest of our results, to our mind, lies in the possibility of easily improving the immunogenicity of any protein or peptide. Furthermore, the incorporation of tag sequences at both extremities of the construction allows the detection of full-length synthesized and secreted proteins by means of immunometric assays (involving commercially available antitag antibodies). Additionally, an advantage of using scFv as a model of low immunogenic protein in this study was that the effects of the T-epitope fusion could be investigated not only on the whole protein synthesis and secretion but also on the possible induction of conformational changes, through functional antigen-antibody measurements.

First, our *in vitro* results strongly suggested that fusion to a T-epitope sequence enhances the synthesis of the recombinant scFv proteins in transfected cells without, however, significantly changing their secretion in the culture medium, the reason for which remaining unclear. Whether this also occurs *in vivo* could not be determined, since no protein (scFv or scFv-T) could be detected in the muscle of any immunized mouse, at least 8 days after the first i.m. injections of plasmids (not shown). In fact, since dendritic cells and macrophages seem to account for the majority of newly expressed proteins after genetic immunization and were shown to migrate rapidly towards the spleen and lymphatic ganglions (Casares *et al.*, 1997; Chattergoon *et al.*, 1998), it would have been interesting to test the possible accumulation, at different time intervals, of newly synthesized scFv or scFv-T in these lymphoid organs. Second, the *in vitro* results illustrated the consequence of fusing even a small-size peptide to a protein on the fine structural conforma-

tion of the latter. Indeed, the fusion of a 16-aminoacid peptide to the C-terminal extremity of the antibody fragment (i.e., in principle remote from the paratope) appeared sufficient to significantly reduce its antigen-binding ability (as revealed by functional assays), probably through allosteric effects. Another explanation would be that the observed enhanced cytoplasmic aggregation of the scFv-T proteins compared to unfused scFv (possibly resulting from a higher synthesis) renders a fraction of scFv-T antibodies usable to recognize their antigen.

Despite this last observation from *in vitro* transfection experiments, the major finding of the present study was actually that *in vivo*, the use of the plasmid coding for the T-epitope fused-scFv protein greatly enhanced the generation of antibodies against conformational epitopes of the undenatured form of the protein. This effect obviously points to an improved antigenic presentation to T-helper lymphocytes, which thus appears to largely compensate for the possible reduced amounts of functional scFv-T proteins (compared to scFv) secreted in extracellular fluids from *in vivo* transfected cells.

Another interesting result was that animals immunized with the T-epitope-containing plasmid also responded by generating high levels of antibodies against the denatured form of the scFv, unlike animals immunized with the unfused scFv encoding vector. This additional result still underlines the improvement of antigen presentation by the T-epitope, but could also suggest that the engagement of the immune response primarily resulted from the lysis of *in vivo* transfected cells, releasing probably higher amounts of cytoplasmic neosynthesized scFv-T (perhaps in aggregates, as discussed above) than of unfused scFv molecules. This latter hypothesis would imply that scFv-cDNA immunizations preferentially enhanced a Th1 response (involving cytolytic T lymphocytes), which, in fact, could be expected from the presence of immunostimulating bacterial se-

TABLE 1. GENERATION OF ANTIBODIES RECOGNIZING A DENATURED FORM OF scFv PROTEINS IN THE SERA FROM ALL MICE IMMUNIZED WITH THE VECTOR CODING FOR THE scFv FUSED WITH THE T EPITOPE (scFv-T)

Mouse no.	Bleeding no.					
	0 preimmune	1	2	3	4	5
11	—	—	—	+	+	—
12	—	—	—	—	+	—
13	—	—	—	—	—	—
14	—	—	—	—	+	—
15	—	—	+	++	+	+

The table summarizes the results from Western blot experiments performed as described in the legend to Figure 3. Immunizations using the plasmid coding for the fusion protein (scFv-T) generated high (+) or very high (++) amounts of anti-scFv antibodies in the sera of four animals out of five, as estimated from the intensity of the band with a molecular weight slightly higher than 30 kDa, the expected size of the scFv (see Fig. 3). Neither serum from any mouse immunized with the unfused scFv cDNA-containing plasmid nor from mice immunized with the control empty vector gave a positive signal in this test (not shown).

quences in the injected plasmids (Davis *et al.*, 1998), and could be investigated further by isotyping the anti-scFv antibodies.

Finally, it should be noted that antinative and antidenatured protein antibodies were found to be produced by different animals or were detected in different bleedings of the same animal (antidenatured scFv then appearing later than antiundenatured scFv antibodies). This observation is in agreement with previous reports (Costagliola *et al.*, 1998), including ours (Tymciuk *et al.*, 2002, although in this case the antidenatured protein antibodies appeared earlier than antinative protein antibodies), but remains difficult to explain. In any case, from a practical point of view, the generation of enhanced levels of both kinds of antibodies still strengthens the interest of this T-epitope fusion procedure since antilinear and anticonformational epitope antibodies are known to exhibit distinct but complementary properties: *in vitro*, they are useful tools for detecting antigens through different techniques (e.g., Western blotting or flow cytometry, respectively) while *in vivo*, upon vaccination for instance, they behave either as cytolysis-inducing antibodies for infected cells expressing pathogen peptide fragments on their membrane or as neutralizing antibodies for extracellular pathogens, respectively.

In conclusion, we have described a simple and efficient method to improve humoral responses against poorly immunogenic proteins in DNA immunizations, based on the fusion of a small-size T-epitope cDNA with the sequence coding for the protein of interest. Taking into account that, at least in protein-based immunizations, this well-defined universal T-epitope derived from tetanus toxin was previously shown (1) to stimulate T cells not only from many mouse strains (Kumar *et al.*, 1992) but also from humans with different genetic (class II MHC) backgrounds (Panina-Bordignon *et al.*, 1989), and (2) to circumvent the problem of tolerance due to epitope-specific suppression in tetanus toxin-primed animals (Kumar *et al.*, 1992; Valmori *et al.*, 1992), the results reported here allow us to expect that this method will also appear of general interest for future applications of DNA-based vaccinations in various species, including humans.

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Address reprint requests to:
Jean-Yves Couraud, Ph.D.

Service de Pharmacologie et d'Immunologie (Bât. 136)
CEA Saclay
91191 Gif-sur Yvette, France

E-mail: couraud@dsvidf.cea.fr

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