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## DNA Fusion Vaccine Designed to Induce Cytotoxic T Cell Responses Against Defined Peptide Motifs: Implications for Cancer Vaccines<sup>1</sup> **FREE**

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*J Immunol* (October,2002)

# DNA Fusion Vaccine Designed to Induce Cytotoxic T Cell Responses Against Defined Peptide Motifs: Implications for Cancer Vaccines<sup>1</sup>

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DNA vaccination offers a strategy to induce immune attack on cancer cells, but tumor Ags are often weak. Inclusion of a “foreign” protein increases immunogenicity, and we found previously that fusion of the fragment C (FrC) of tetanus toxin to the tumor Ag sequence promotes Ab and CD4<sup>+</sup> responses against B cell tumors. For CTL responses, use of the full two-domain FrC may be less helpful, because known immunogenic MHC class I-binding peptides in the second domain could compete with attached tumor-derived epitopes. Therefore, we removed the second domain, retaining the N-terminal domain, which contains a “universal” helper epitope. We investigated the ability to induce CTL responses of candidate peptides placed at the C terminus of this domain. As test peptides, we repositioned the two known CTL motifs from the second domain to this site. Strong CTL responses to each peptide were induced by the engineered construct, as compared with the native FrC construct. Induced CTLs were able to specifically kill tumor cells transfected with FrC as a surrogate tumor Ag both in vitro and in vivo. Further reduction of the domain to a short helper epitope generated only weak CTL responses against fused peptides, and synthetic peptides mixed with the plasmid containing the first domain were ineffective. The single FrC domain-peptide vaccine design also was able to induce high levels of CTLs against a known epitope from carcinoembryonic Ag. Response to peptide was suppressed if two FrC domains were present, consistent with immunodominance. These principles and designs may have relevance for cancer vaccines delivered via DNA. *The Journal of Immunology*, 2001, 167: 1558–1565.

Strategies for activating immunity against cancer are now under intensive investigation. DNA vaccination offers a relatively simple route between identification of genetic changes in tumor cells and preparing a test vaccine. However, the way in which the encoded protein gains access to the machinery of Ag presentation and to activation of effective immunity is not yet completely clear. Injected muscle cells do not appear to present Ag directly to T cells (1, 2), but are likely to act as a reservoir of Ag for indirect presentation (2). Direct transfection of APCs may occur from the muscle site (3), but only at very low levels (4, 5). There is also the question of which effector pathway will be most efficient in attacking the tumor cell, and this obviously depends on the nature of the expressed Ag.

We have been developing DNA vaccines to treat B cell malignancies, using as the target Ag the idiotypic (Id)<sup>3</sup> determinants of the clonotypic Ig, encoded by the variable region genes V<sub>H</sub> and V<sub>L</sub> (6, 7). For lymphoma, anti-Id Ab is effective in killing tumor cells (6, 8, 9); therefore, our DNA vaccine was designed to induce Ab. Initially, a vaccine containing the V<sub>H</sub> and V<sub>L</sub> genes assembled as single-chain fragment variable region of Ig (scFv) alone (10)

proved ineffective in inducing anti-Id Ab in mouse models (11). Fusion of a gene encoding the fragment C (FrC) of tetanus toxin (TT) to the scFv sequence led to strong promotion of Ab production with protection against lymphoma challenge (12, 13). This design is now being tested in a pilot clinical trial of patients with low grade follicular lymphoma. The requirement for fusion of genes encoding additional proteins, such as xenogeneic protein (14) or chemokines (15), to engage the immune response against Id Ags has been a general finding. In our case, the fact that fusion was an absolute requirement, with separate plasmids having no promotional effect, supported the concept that the FrC-specific T cells may be providing help to B cells secreting anti-Id Ab (12). Interestingly however, the same scFv-FrC design was able to induce protective immunity against an Ig-secreting, surface Ig-negative myeloma model, apparently mediated by effector T cells (13) and likely to be of the CD4<sup>+</sup> subset (16).

Although this design may be suitable for surface or secreted target Ags, many candidate tumor Ags are intracellular and will be presented only as peptides in association with MHC class I molecules (reviewed in Ref. 17). The question then is whether fusion with FrC sequence would be necessary or useful for inducing CTL-mediated immunity against candidate tumor-derived peptides. We had already found that FrC itself, when delivered as a DNA vaccine, was able to induce a CTL response, and an H2-K<sup>b</sup>-restricted peptide motif had been identified at position 1287–94 in the FrC sequence (13, 18). The phenomenon of immunodominance, in which CD8<sup>+</sup> T cells focus on only one or a few peptide motifs, is clearly evident in responses to viral infection (19). In fact, immunodominance has been described as a central feature of CD8<sup>+</sup> T cell responses (reviewed in Ref. 20). If this is the case for DNA vaccines, it would argue against fusing potentially competing FrC sequence to the tumor peptide sequence.

FrC is composed of two domains, a jelly roll N-terminal domain and a second  $\beta$ -trefoil domain (21). The first domain contains a

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<sup>3</sup> Abbreviations used in this paper: Id, idiotypic; scFv, single-chain fragment variable region of Ig; FrC, fragment C; TT, tetanus toxin; CEA, carcinoembryonic Ag; BCL, B cell lymphoma; f, forward; r, reverse; ER, endoplasmic reticulum; SEV, Sendai virus nucleoprotein.

well-described “universal” helper epitope, p30 (22, 23), which binds to a range of mouse and human MHC class II alleles and is recognized by CD4<sup>+</sup> T cells (24). Previously, we identified an epitope involved in inducing CD8<sup>+</sup> T cells in the second domain (18). We have identified a further epitope with a similar ability to induce CD8<sup>+</sup> T cell responses also in this domain. We have now investigated two factors that may be important for induction of CTL responses against candidate epitopes presented via DNA: first, the position of the peptide epitope in the DNA sequence; and, second, the role of the domain containing the helper epitope in promoting CTL activity. To test the relevance of the induced CTLs for attacking cancer cells, we have transfected full-length FrC into EL-4 cells, where processed peptides can act as surrogate target Ags. To move closer to cancer, we have also demonstrated that a vaccine of similar design and incorporating a known epitope from carcinoembryonic Ag (CEA) induced high levels of specific CTL. Using this model, the requirement to remove potentially competing epitopes from the adjuvant FrC sequence was validated.

## Materials and Methods

### Construction of DNA vaccines

Construction of the DNA vaccine (p.FrC) containing the gene encoding the full-length two-domain sequence of FrC (aa 865–1316 of TT (TT<sub>865–1316</sub>)), with a leader sequence derived from the V<sub>H</sub> of the IgM of the B cell lymphoma (BCL)1 tumor has been previously described (25, 26). The DNA vaccine containing the gene encoding the first domain (21) (p.DOM) was constructed by PCR amplification of the N-terminal domain sequence (TT<sub>865–1120</sub>) from p.FrC using the forward (f) and reverse (r) primers FrCf1 and FrCr1, respectively (Table I), before cloning into pcDNA3. This plasmid was then used as template for the construction of three similar vaccines, each including the first domain but with a distinct CTL epitope sequence fused to the C terminus. Assembly of p.DOM-peptide 1, encoding the TT<sub>1287–1294</sub> peptide; p.DOM-peptide 2, encoding the TT<sub>1162–1169</sub> peptide; or p.DOM-CEA, encoding the CEA<sub>526–533</sub> peptide, was identical with that of p.DOM alone, except that a different reverse primer was used in each case (FrCr2, FrCr3, and FrCr4, respectively), which overlapped with the p.DOM carboxyl sequence and incorporated the CTL epitope sequence of interest. The DNA vaccine p.FrC-CEA<sub>526–533</sub> was constructed by PCR amplification of the full-length FrC sequence using the forward primer FrCf1, together with primer FrCr5, which overlaps the 3' sequence of FrC and encodes the CEA CTL epitope (CEA<sub>526–533</sub>), fusing it to the C terminus of FrC. The PCR product was then cloned into pcDNA3.

The p.p30-peptide 1 vaccine was assembled by linking the DNA sequence encoding the CTL epitope TT<sub>1287–1294</sub> to that encoding a universal T helper epitope, p30 (TT<sub>947–967</sub>), located in the first domain of FrC (24). A three-step PCR assembly procedure was used: First, the BCL<sub>1</sub> leader sequence was amplified from p.FrC using the forward primer PCMVf1, together with the reverse primer BCL1r1 containing p30 overlapping sequence. Second, p30 was amplified from p.FrC with the forward primer p30f1 and the reverse primer p30r1 containing an overhang encoding the CTL epitope TT<sub>1287–1294</sub>. Third, these two gel-purified PCR products were combined and assembled by PCR SOEing (combining and extending overlapping sequences) using primers PCMVf1 and p30r1. The vaccine p.p30-

peptide 2 was assembled in a similar manner using the reverse primer p30r2 containing an overhang encoding the CTL epitope TT<sub>1162–1169</sub>. All assembled vaccine PCR products were ligated into the expression vector pcDNA3 (Invitrogen, San Diego, CA) using *Hind*III and *Not*I restriction sites. Primer sequences are shown in Table I.

The structures of the DNA vaccines are indicated in Fig. 1. Integrity of all constructs was confirmed by DNA sequencing. Expression and size was checked *in vitro* using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI). Expression in mammalian cells was tested by transfecting COS cells and measuring FrC-containing protein in the supernatant by ELISA (12).

### Peptides

FrC peptides were synthesized in house on a Shimadzu PSSM8 peptide synthesizer (Shimadzu Scientific Instruments, Columbia, MD) using Fmoc chemistry and were checked for purity by HPLC. Concentrations were measured by a colorimetric assay (BCA; Pierce, Rockford, IL). The coordinates for the H-2<sup>b</sup>-restricted FrC CTL epitope sequences TT<sub>1287–1294</sub> (SNWYFNHL;peptide 1) and TT<sub>1162–1169</sub> (LNYYRRL;peptide 2) correspond to the complete TT sequence. The CEA<sub>526–533</sub> peptide (EAQNTTYL) has been described previously (27, 28). It was synthesized commercially and supplied at >95% purity (Peptide Protein Research, Southampton, U.K.).

### Peptide binding assay

Binding of each peptide to H2-K<sup>b</sup> was performed using the assembly assay as described (29). This assay is based on the observation that, in a detergent lysate of RMA-S cells, K<sup>b</sup> molecules are unstable and dissociate after an overnight incubation at 4°C unless a stabilizing (K<sup>b</sup>-binding) peptide is added at the time of lysis. Therefore, only stabilized K<sup>b</sup> molecules can be recovered by immunoprecipitation with mAb Y3 after overnight incubation. The amount of recovered K<sup>b</sup> is directly proportional to the amount of peptide bound, and the concentration of peptide required to effect 50% maximal recovery represents an approximate binding affinity (29). Recovery of H2-K<sup>b</sup> H chains was quantitated after immunoprecipitation and SDS-PAGE using AIDA (Fuji, Tokyo, Japan).

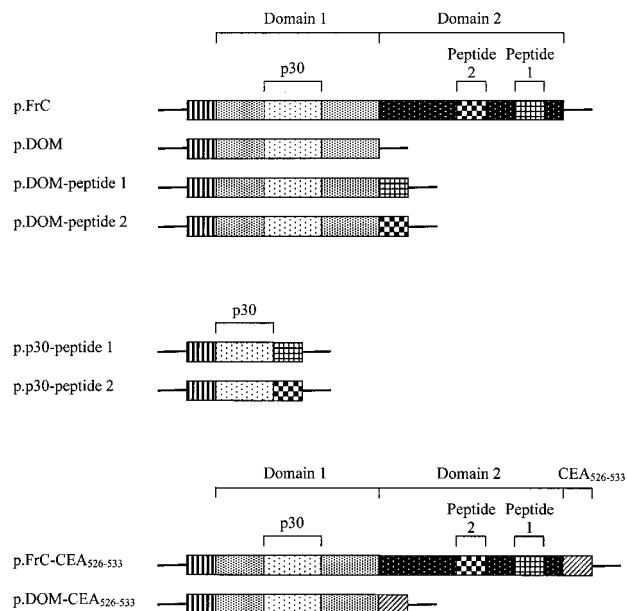
### Vaccination protocol and CTL assay

C57BL/6 mice, bred in house, were vaccinated at 6–10 wk of age with 50 μg DNA in normal saline injected into two sites in the quadriceps muscles. For measurement of CTL responses, mice were sacrificed on day 14. Spleens were pooled from vaccinated mice, and single-cell suspensions were prepared in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Life Technologies, Paisley, U.K.), 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential amino acids (1% of 100× stock), 25 mM HEPES buffer, and 50 μM 2-ME. Splenocytes were resuspended in 40 ml medium at 3 × 10<sup>6</sup> cells/ml and added to 80-cm<sup>2</sup> flasks along with recombinant human IL-2 (20 U/ml, PerkinElmer, Foster City, CA) and peptide (5–20 μM). In some indicated experiments, T cell cultures were re-stimulated 7 days later in 24-well plates. T cells (5 × 10<sup>5</sup>/well) were mixed with irradiated syngeneic “feeder” splenocytes (5 × 10<sup>6</sup>/well) together with rIL-2 (20 U/ml) and peptide (5–20 μM). Cytolytic activity of the T cell cultures was generally assessed 6 days after one *in vitro* stimulation by standard 4- to 5-h <sup>51</sup>Cr release assays, as previously described (18). Target cells were EL4 cells (American Type Culture Collection, Manassas, VA; TIB 39) incubated with a test or control peptide, EL4 cells alone, or transfected EL4 cells (see below). Specific lysis was calculated by the standard

Table I. Oligonucleotide primers used to assemble vaccine constructs (5' to 3')

Primer	Sequence <sup>a</sup>
FrCf1	TTTTAAGCTTGCCGCCACCATGGGTTGGAGC
FrCr1	AAAAGCGGCCGCTTAGTTACCCAGAAAGTCACGCAG
FrCr2	AAAGCGGCCGCTTACAGGTGGTTGAAGTACCAGTTAGAGTTACCCAGAAAGTCACGCAG
FrCr3	AAAGCGGCCGCTTACAGACGTCGGTAGTAGATGTTACAGTTACCCAGAAAGTCACGCAG
FrCr4	AAAGCGGCCGCTTACAGGTAGGTTGTGTTCTGAGCCTCGTTACCCAGAAAGTCACGCAG
FrCr5	TTTGGCGCCGCTTACAGGTAGGTTGTGTTCTGAGCCTCGTCGTTGGTCCAACCTTCATCGGT
PCMVf1	CTATATAAGCAGAGCTCT
BCL1r1	GGTGAAGTTGTTGAAGGAGTGCACACCTGTAGCTGT
p30f1	ACAGGTGTGCACTCCTTCAACAACCTTACCCGTTAGC
p30r1	AAAGCGGCCGCTTACAGGTGGTTGAAGTACCAGTTAGATTCCAGGTGGGAAGCAGAAAC
p30r2	AAAGCGGCCGCTTACAGACGTCGGTAGTAGATGTTACGTTCCAGGTGGGAAGCAGAAAC

<sup>a</sup> Restriction enzyme sites are underlined.



**FIGURE 1.** Schematic diagram indicating DNA vaccine design. Vaccine sequences were assembled and inserted into pcDNA3 using *Hind*III and *Not*I restriction enzyme sites. DNA sequences included those encoding the two domains of full-length FrC (■ and ▣) or the amino-terminal domain only (□), p30 (▤), BCL1 leader (▥), TT<sub>1287–1294</sub> (peptide 1, ▦), TT<sub>1162–1169</sub> (peptide 2, ▧), and CEA<sub>526–533</sub> (▨). The DNA sequence encoding the Th epitope p30 is in the p.DOM sequence (▩); the H-2K<sup>b</sup>-restricted CTL epitopes TT<sub>1287–1294</sub> (peptide 1) and TT<sub>1162–1169</sub> (peptide 2) are in the second (▫) domain.

formula ( $[(\text{release by CTL} - \text{release by targets alone}) / (\text{release by 4\% Nonidet P-40} - \text{release by targets alone})] \times 100\%$ ). Spontaneous release by targets alone was always  $<20\%$  of release by 4% Nonidet P-40.

#### Intracellular IFN- $\gamma$ assay

Viable cells were selected by density centrifugation (Lymphoprep; Nycomed, Oslo, Norway). T cells were incubated for 4 h at 37°C in 96 U-well plates at  $5 \times 10^5$  cells/well together with 10 U/well rIL-2, 1  $\mu\text{M}$  peptide, and 1  $\mu\text{l}$ /well GolgiPlug (BD PharMingen, San Diego, CA). Cells were blocked with 2% de complemented mouse serum (15 min, 4°C) before labeling with 1  $\mu\text{g}$ /well FITC-labeled anti-mouse CD8b.2 (Ly-3.2, clone 53-5.8; BD PharMingen) or an isotype control (20 min, 4°C). Following surface labeling, the cells were fixed with 1% formaldehyde (20 min, 4°C) and then permeabilized with 0.5% saponin (10 min, 4°C) before intracellular labeling with 0.5  $\mu\text{g}$ /well PE-labeled rat anti-mouse IFN- $\gamma$  (clone XMGI.2; BD PharMingen) for 20 min at 4°C. After a final wash, the cells were resuspended in PBS and analyzed immediately by FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

#### Tumor targets

We have generated a tumor model consisting of EL4 tumor cells into which we have transfected a plasmid encoding a nonsecreted (leaderless) form of FrC (18). Briefly,  $2 \times 10^6$  cells in 400  $\mu\text{l}$  medium were mixed with 10  $\mu\text{g}$  plasmid DNA and electroporated at 300 V, 975  $\mu\text{F}$  (Gene Pulser Cupvette, 0.4-cm electrode gap; Bio-Rad, Hercules, CA). The cells were grown in the presence of a selective antibiotic (2 mg/ml geneticin; Life Technologies) and, following the restoration of a stable population, were cloned and tested for susceptibility to lysis by FrC-specific CTLs. This led to the generation of the tumor cell line EL4-FrC.

#### Tumor challenge

C57BL/6 mice were challenged by s.c. injection of  $1 \times 10^5$  EL4-FrC transfectants or EL4 cells transfected with empty vector (pcDNA3) into the right flank. Mice were sacrificed when the resulting tumor reached 1.5 cm in diameter, in accordance with humane endpoint guidelines (U.K. Coordinating Committee for Cancer Research, London, U.K.), and the day of death was recorded. Cell depletion experiments were performed *in vivo* by i.p. injection of 100  $\mu\text{g}$  Ig (rat anti-mouse CD8, YTS 169.4.2.1, kindly

supplied by Dr. S. Cobbold, Sir William Dunn School of Pathology, Oxford, U.K.; Ref. 30) or an isotype control, which were given every 2–3 days for 14 days beginning 1 wk before tumor challenge.

## Results

### Identification of CTL-inducing MHC class I binding motifs in FrC

The amino acid sequence of FrC was scanned for peptide 8-mer motifs with potential for binding to H2-K<sup>b</sup> or H2-D<sup>b</sup> (31). Using an algorithm to assign a score based on the estimated half-time of dissociation of a molecule containing this sequence (31), eight peptides with values of  $>13$  for binding to H2-K<sup>b</sup> were identified and synthesized. The top 20 predicted binding sequences gave values ranging from 86.4 to 1.32. Known immunodominant K<sup>b</sup>-restricted CTL epitopes score between 132 (RGYVYQGL) and 17 (SIINFEKL). The K<sup>b</sup>-binding Sendai virus nucleoprotein (SEV)-derived sequence FAGNYPAL scored 60, whereas a control D<sup>b</sup>-restricted epitope (ASNENMDAM) scored 1. FrC sequences that scored  $<8$ , including the only H2-D<sup>b</sup>-binding candidate, were not investigated further. Mice were then vaccinated with the DNA vaccine containing the gene encoding two-domain FrC (p.FrC). At day 14 after one vaccination, CTL responses against peptide-loaded EL4 cells could be detected using two of the eight peptides following one restimulation *in vitro* (Fig. 2*a*). Further injections or additional restimulations *in vitro* failed to elicit CTL responses to the remaining six candidate peptides. The two positive peptides, which we have termed peptide 1 and peptide 2, were derived from the second domain of FrC, sited at positions 1287–1294 and 1162–1169, respectively.

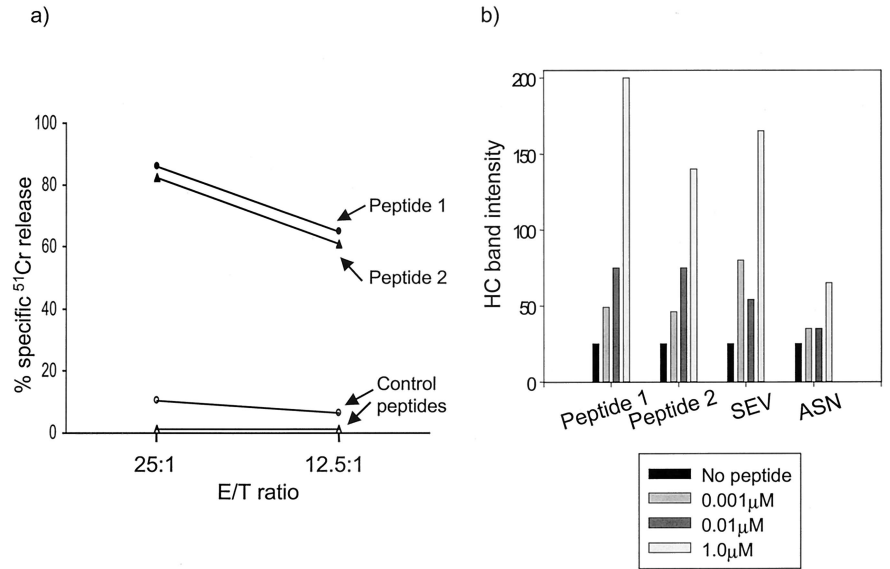
The remaining peptides were unable to stimulate any detectable CTL activity. Interestingly, of the two immunostimulatory peptides, peptide 1 (SNWYFNHL) had the lowest score (13.2), and peptide 2 (LNIYYRRL) was ranked third, with a score of 26.4. In an *in vitro* binding assay, both peptide 1 and peptide 2 were able to bind to H2-K<sup>b</sup> equally well and not significantly differently to the positive control peptide SEV (Fig. 2*b*). Thus, although the predictive algorithm was successful in identifying both immunostimulatory sequences, there may be poor correspondence between predicted binding and the actual ability to bind to class I and stimulate a CTL response, as has been noted before (32).

### Effect of repositioning the peptide sequences to the C terminus of the first domain

Peptides 1 and 2 were able to induce CTL responses following vaccination with full-length FrC sequence, but the response was relatively weak, with two restimulations required to produce high levels of <sup>51</sup>Cr release. Because tumor Ags may also have low immunogenicity, we used these peptides as models to improve immunogenic activity via DNA delivery. We investigated first the effect of removing the peptide sequences from the FrC backbone and repositioning them at the C terminus of the first domain (p.DOM). A single i.m. vaccination with p.DOM-peptide 1 or p.DOM-peptide 2 generated rapid high-level CTL responses detectable after one stimulation *in vitro*. Comparison with the original two-domain p.FrC vaccine is shown in Fig. 3, and similar results were obtained in multiple experiments.

Cytolytic activity was paralleled by the levels of intracellular IFN- $\gamma$  found in the CD8<sup>+</sup> T cell population (Fig. 4, *a* and *b*), with the p.DOM-peptide vaccines regularly inducing 2- to 3-fold increases in the percentages of IFN- $\gamma$ -positive CD8<sup>+</sup> cells as compared with the p.FrC vaccine. The CTLs were also able to reproducibly lyse EL4 cells transfected with leaderless p.FrC, with an E:T ratio of 30:1, giving values of 13- and 8%-specific <sup>51</sup>Cr release

**FIGURE 2.** CTL responses induced by vaccination with DNA encoding full-length FrC sequence (p.FrC) are specific for two H2-K<sup>b</sup>-binding octamers. Following vaccination with p.FrC, splenocytes taken at day 14 were restimulated with each of eight peptides with significant predicted H-2K<sup>b</sup>-binding activity. Only two peptides induced measurable CTL activity as measured by <sup>51</sup>Cr release assay. *a*, CTL activity against peptide-loaded EL4 cells was detected against peptide 1 (SNWYFNHL) and peptide 2 (LNIYYRRL). Each peptide was used reciprocally as either test or control. *b*, Binding activity to H-2K<sup>b</sup> by a stabilization assay showed that peptides 1 and 2 were comparable to the positive control from SEV and were clearly distinct from the control H-2D<sup>b</sup>-binding peptide from influenza virus (ASN).

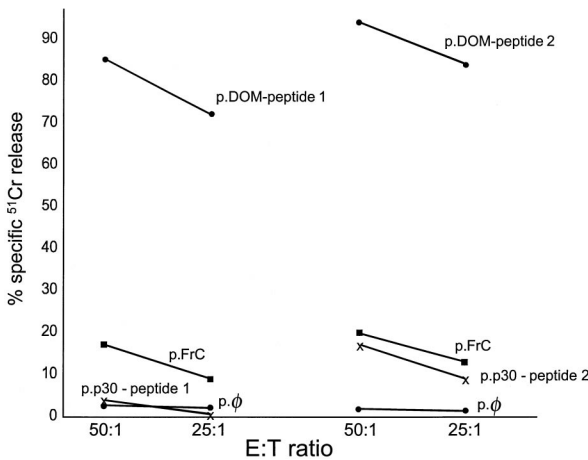


for CTLs against peptide 1 and peptide 2, respectively, in a representative experiment. Although these levels were low, they were consistent in repeated experiments. In contrast, no significant lysis (<1.5%-specific <sup>51</sup>Cr release) of EL4 cells transfected with empty vector was observed. Addition of peptide to the target cells clearly increased specific lysis, indicating that the transfectant was able to process and present only low levels of both peptides by the endogenous route (data not shown). However, levels of expression were sufficient for effector CTLs specific for either peptide 1 or 2 to attack the transfectant in vivo (see below).

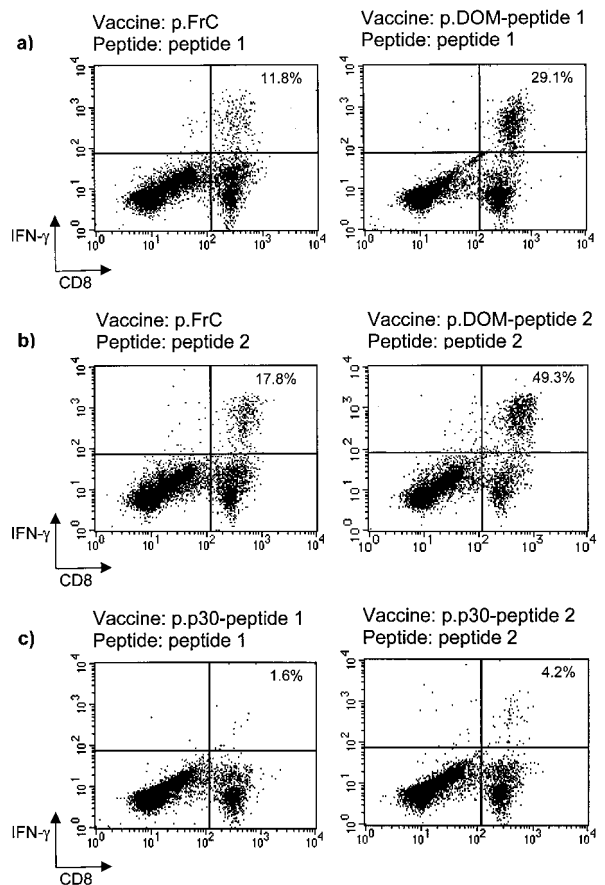
*Contribution of domain 1 (p.DOM) to CTL induction via DNA vaccination*

Domain 1 contains one identified universal peptide at position 947–967, which can be recognized by human T cells (24) or mouse

T cells (33) in association with a large number of MHC class II molecules. Because this could be a critical component of p.DOM for provision of T cell help (34), we investigated its role in CTL induction. We compared the ability of a DNA vaccine containing



**FIGURE 3.** Repositioning peptides 1 or 2 from the embedded site in the second domain to the C terminus of the first domain of FrC amplifies the specific anti-peptide CTL responses. Vaccination with DNA encoding either full-length FrC (p.FrC), the first domain fused to peptide 1 or 2 repositioned at the C terminus (p.DOM-peptide), the p30 helper epitope of FrC fused to peptide 1 or 2 sequence (p.p30-peptide), or control plasmid containing no insert (p.φ) was conducted. At day 14, splenocytes were restimulated with peptide 1 (left side) or peptide 2 (right side) for 6 days in vitro before measuring CTL activity by a <sup>51</sup>Cr release assay using peptide-loaded EL4 target cells. Lysis of target cells loaded with control peptide was negligible in each case (<2.2%). Representative data are shown from similar results obtained in three of three experiments.



**FIGURE 4.** Comparison of the abilities of DNA vaccines containing embedded or repositioned peptides to induce CD8<sup>+</sup> T cells containing intracellular IFN-γ. In parallel with the <sup>51</sup>Cr release assay (Fig. 3), FACS analysis was used to measure the percentages of CD8<sup>+</sup> T cells containing intracellular IFN-γ. The relative efficiencies of the constructs paralleled the <sup>51</sup>Cr release assays (Fig. 3). Representative data are shown from similar results obtained in two of two experiments.

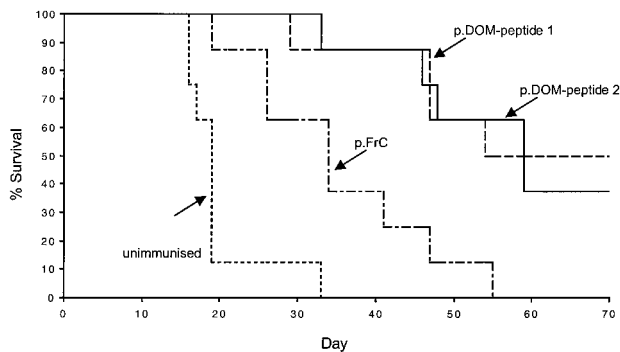
only the p30 sequence linked to each of the CTL peptide sequences with that of p.DOM-peptide vaccines. Fig. 3 shows that p.p30-peptide 1 was poor in inducing a CTL response; p.p30-peptide 2 was more effective, but it performed considerably less well than p.DOM-peptide 2 and was, in fact, less effective than the original two-domain p.FrC vaccine. A comparison of the numbers of CD8<sup>+</sup> T cells producing intracellular IFN- $\gamma$  showed the same trend (Fig. 4c). Repeated vaccination and restimulation with the p.p30-peptide vaccines could generate CTLs (data not shown) confirming the integrity of the constructs, but indicating their inferior performance. The conclusion is that p.DOM contains additional sequence information required for induction of an effective CTL response against attached peptides.

#### Contribution of p.DOM to CTL induction via peptide vaccination

We then investigated whether the adjuvant effect of p.DOM on CTL induction via DNA delivery was apparent when administered with a synthetic peptide. Peptide 1 was mixed with p.DOM and injected into muscle. However, no CTL activity was induced, even following three injections at days 0, 21, and 42 and up to four weekly restimulations *in vitro* (data not shown). It appears that fusion of p.DOM to the peptide sequence is required, either for delivery to the same cell or to ensure that synthesis of the first domain and the presence of the peptide are coincident.

#### Protection

Vaccination at days 0 and 21 with p.DOM-peptide 1 or with p.DOM-peptide 2 led to significant protection against challenge with the EL4-FrC transfectant at day 28 (Fig. 5), with no effect on growth of EL4 cells transfected with empty vector (pcDNA3) (data not shown). At this relatively early time of challenge (day 28), the p.DOM-peptide vaccines were superior to the two-domain p.FrC



**FIGURE 5.** Comparison of the abilities of DNA vaccines containing embedded or repositioned peptides to induce protective immunity against challenge with EL4-FrC-transfected tumor cells. Vaccination with DNA encoding either full-length FrC (p.FrC), the first domain fused to peptide 1 sequence repositioned at the C terminus (p.DOM-peptide 1), or the first domain fused to peptide 2 sequence repositioned at the C terminus (p.DOM-peptide 2) was conducted on days 0 and 21 using eight mice per group. At day 28, 10<sup>5</sup> tumor cells were injected *s.c.*, and mice were sacrificed when tumor size reached 1.5 cm in diameter. Data from one of two experiments with similar results are shown. The unimmunized p.FrC and p.DOM-peptide 1 groups were tested together. p.FrC and p.DOM-peptide 1 both induced significant protection ( $p < 0.01$ , Yates' continuity correction) compared with unimmunized controls; p.DOM-peptide 1 significantly improved the level of protection compared with p.FrC ( $p < 0.02$ ). The p.DOM-peptide 2 vaccine was tested separately on age- and sex-matched mice and was found to induce significant protection ( $p < 0.01$ ) comparable to p.DOM-peptide 1 in two of two experiments. To simplify the presentation, we have therefore combined the data.

plasmid, consistent with the rapid induction of CTLs observed *in vitro*. However, although the p.FrC vaccine failed to generate sufficient CTLs by day 14 to kill the transfectant *in vitro* after one restimulation, some protection was evident (Fig. 5), likely due to expansion of CTLs by the second injection. CTLs able both to kill the transfectant *in vitro* and to protect against challenge could be induced by the two-domain p.FrC after a third vaccine injection (data not shown). Vaccination with the plasmids containing only the p30 helper epitope fused to either the peptide 1 or 2 sequence was completely ineffective in providing protection, as expected from the poor ability to induce CTLs (data not shown). Depletion experiments showed that all protection was abrogated by depletion of CD8<sup>+</sup> T cells (data not shown). Depletion of CD4<sup>+</sup> T cells could not be conducted due to expression of CD4 by EL-4 cells. These results indicate that the CTLs induced by the repositioned peptides are contributing to protection against tumor.

#### p.DOM-peptide design to induce CTLs against a peptide from CEA

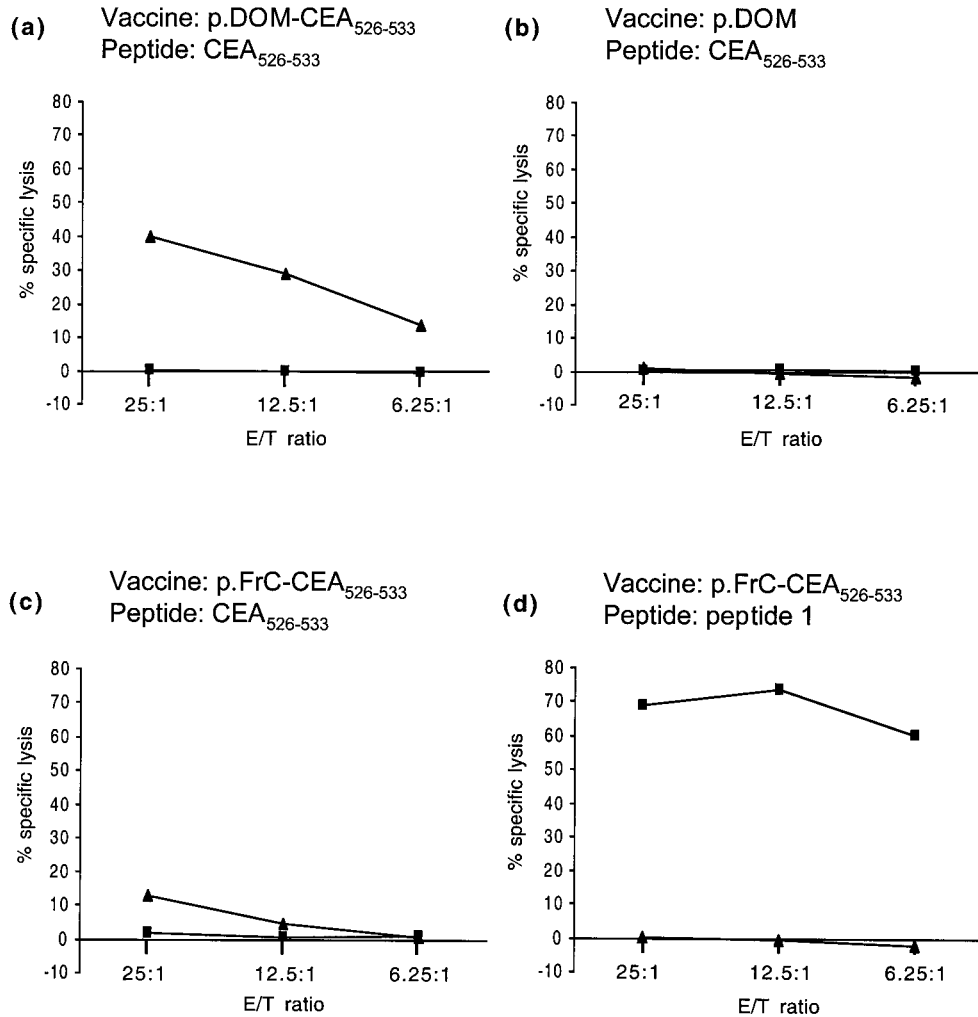
To test the ability of the p.DOM-peptide design to induce CTLs against a candidate tumor-associated Ag, a peptide derived from human CEA was chosen. Peptide EAQNTTYL is known to act as a target for CTLs induced by vaccination of C57BL/6 mice with recombinant vaccinia virus (27). The encoding sequence was placed at the 3' end of the first domain to make the p.DOM-CEA peptide vaccine (Fig. 1). This was injected into mice, and CTL activity was measured on day 14 after one restimulation *in vitro*. Significant and reproducible (three experiments) levels of cytolytic activity were induced (Fig. 6a) with ~19% of IFN- $\gamma$ -containing CD8<sup>+</sup> T cells (Fig. 7a).

#### Evidence for epitopic competition

The CEA model was used to investigate the assumption that epitopes in the second domain of FrC would compete with attached tumor-derived epitopes. The CEA peptide sequence was placed at the carboxyl end of the two-domain (full-length) FrC sequence to produce p.FrC-CEA<sub>526-533</sub> (Fig. 1). The ability of this construct to induce CEA-specific CTLs was then compared with that of the single domain p.DOM-CEA<sub>526-533</sub> design, using one injection and one restimulation *in vitro*. In repeated experiments, the single domain vaccine induced 2- to 3-fold higher levels of CTL activity against the CEA epitope (Fig. 6a) as compared with the construct containing two-domain FrC (p.FrC-CEA<sub>526-533</sub>) (Fig. 6c). However, the two-domain construct was able to induce high levels of CTL activity against the FrC peptide 1 (Fig. 6d). This strongly suggests that inclusion of potentially competitive epitopes within the second domain of FrC leads to suppression of induction of CEA-specific CTL activity.

Cytolytic activity against CEA peptide was paralleled by the levels of intracellular IFN- $\gamma$  found in the CD8<sup>+</sup> T cell population, with the p.DOM-CEA<sub>526-533</sub> vaccine inducing 2- to 3-fold higher levels of IFN- $\gamma$ -positive cells as compared with the two-domain vaccine (Fig. 7, a and c). As expected, the induction of high levels of CTL activity against peptide 1 of the second domain of FrC was mirrored by high levels of IFN- $\gamma$ -positive CD8<sup>+</sup> T cells (Fig. 7d). The control p.DOM vaccine produced no significant CTL activity (Fig. 6b) and very low levels of IFN- $\gamma$ -positive CD8<sup>+</sup> T cells (Fig. 7b).

These results confirm the anticipated advantage of removing the second domain of FrC on induction of the response to the CEA epitope and suggest that the p.DOM-peptide design may have general application for tumor Ags.



**FIGURE 6.** Suppression of the CTL response against a candidate peptide from CEA using the two-domain FrC-peptide design is overcome by using the p.DOM-peptide design. Vaccination with DNA encoding the first domain of FrC with a sequence encoding the EAQNTTYL peptide from CEA fused to the C terminus (p.DOM-CEA<sub>526-533</sub>) (a), DNA encoding the first domain of FrC alone (p.DOM) (b), or DNA encoding the full-length FrC with the sequence encoding the EAQNTTYL peptide fused to the C terminus (p.FrC-CEA<sub>526-533</sub>) (c and d). At day 14, splenocytes were restimulated with CEA<sub>526-533</sub> (a–c) or peptide 1 (d) for 6 days in vitro before measuring CTL activity by a <sup>51</sup>Cr release assay using EL4 target cells loaded with either CEA<sub>526-533</sub> peptide (▲) or peptide 1 (■). Representative data are shown from similar results obtained in three of three experiments.

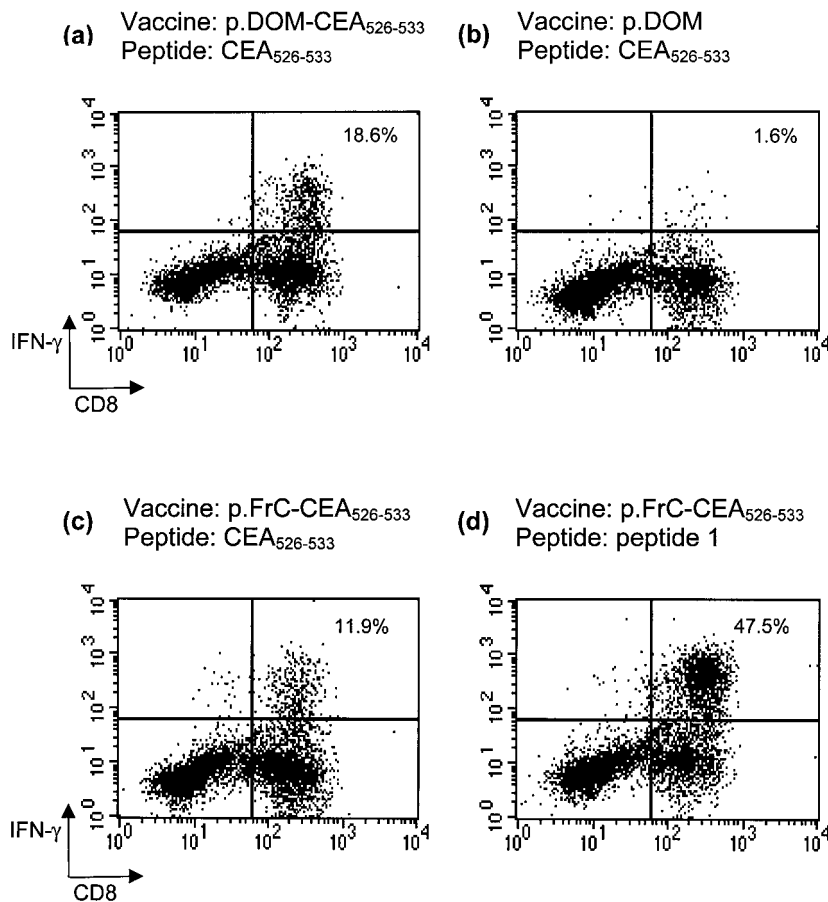
## Discussion

Successful vaccination against cancer is likely to require activation of multiple pathways of immunity, including CTLs. Although DNA vaccines are efficient in inducing CTL responses (35), tumor Ags are often weak (12), and deletion of high affinity CD8<sup>+</sup> T cells may have occurred (36). To generate CTLs, candidate peptides should be processed and presented efficiently by the APCs, preferably in the absence of competing peptides that could override the response (reviewed in Ref. 20). We have investigated CTL responses against the FrC sequence of TT, first because of our interest in using FrC as an adjuvant “foreign” sequence to activate immunity against attached scFv sequence (12). Using our DNA scFv-FrC vaccines against a mouse B cell lymphoma and myeloma, protective anti-Id immunity is promoted involving Ab and CD4<sup>+</sup> T cells, respectively (13). CTL responses were not detected against scFv, possibly due to a lack of MHC class I-binding motifs within the V genes. However, CTLs were induced against two peptide motifs in the second domain of FrC (13, 18). Although responses were relatively weak, they have the potential to compete with CTL motifs from attached tumor sequences (37). To investigate the adjuvant potential of FrC sequence for inducing CTLs

against candidate peptides, we removed the second domain, leaving the first domain to provide T cell help via the universal peptide p30 (24, 34).

Operation of the first domain (p.DOM) as an adjuvant was then tested using the two peptide motifs from the second discarded domain. Repositioning to the C terminus of p.DOM led to a striking increase in CTLs against each peptide. We found previously that CTLs against FrC are induced more efficiently if the leader sequence is present (18), and consequently, we have included the leader in all the constructs described here. Inclusion of the leader sequence should ensure that all constructs longer than ~60 aa are cotranslationally transported into the endoplasmic reticulum (ER). The effect of repositioning may then reflect the “C-end rule,” whereby antigenic peptides are preferentially produced from the C terminus of precursor peptides or proteins in the ER site (38). This could also be relevant for indirect transfer of peptides from muscle to APCs via heat shock proteins such as gp96 or calreticulin, which are normally resident in the ER (39).

The second investigation was to assess the contribution of p.DOM to induction of CTLs against attached peptide sequence. In a model system, a DNA vaccine encoding the immunogenic K<sup>B</sup>-



**FIGURE 7.** Comparison of the abilities of the two-domain FrC-peptide design and the p.DOM-peptide design to induce CD8<sup>+</sup> T cells containing intracellular IFN- $\gamma$ . In parallel with the <sup>51</sup>Cr-release assay (Fig. 6), FACS analysis was used to measure the percentages of CD8<sup>+</sup> T cells containing intracellular IFN- $\gamma$ . The relative efficiencies of the constructs paralleled the <sup>51</sup>Cr-release assays (Fig. 6). Representative data are shown from similar results obtained in three of three experiments.

restricted epitope of OVA (SIINFEKL) fused to the adjacent I-A<sup>b</sup>-restricted helper peptide sequence was able to induce CTLs (34). This indicates that a helper epitope and a CTL epitope might be the only requirements. In our case, this design was insufficient, because a fusion gene encoding the universal helper peptide sequence fused to either of the CTL epitopes generated only low levels of CTL activity. This may be due to the relative weakness of the FrC-derived peptides as compared with the OVA peptide, but it may indicate a problem for tumor Ags. Another possibility relates to the important contribution of the leader sequence to the immunological potency of these DNA vaccines. The supply of shorter constructs to the ER may be poor because these will depend on posttranslational rather than cotranslational transport (40), which is less efficient and may therefore give rise to reduced priming. Nevertheless, it is likely that additional sequences in p.DOM either provide more T cell help or contribute to peptide presentation by other mechanisms. One possibility is that the 25-kDa domain increases the level of the attached peptide by protecting it from degradation in the cytosol. A further possibility is that the presence of some misfolded FrC domain in the ER may influence loading of attached peptides onto heat shock proteins for cross-priming (41).

The finding that p.DOM-peptide 1 activates a rapid CD8<sup>+</sup> T cell-mediated protective immunity against the EL4-FrC transfectant and appears more efficient than the vaccine containing full-length two-domain FrC (p.FrC) indicates two features with relevance for cancer therapy. The first is that repositioning can increase the effectiveness of a vaccine aimed to induce peptide-specific CTLs. Obviously, the peptide chosen must also be presented by the tumor cell, but levels required for effector cell recognition can be low (20). The second is that p.DOM can provide

activating signals required for DNA vaccines against weak peptide Ags. Interestingly, four of six candidate HLA-A2-binding motifs are also in the second domain of FrC, and early studies have shown that the highest functional levels are also in that sequence (J. Rice, T. Elliott, S. Buchan, F. K. Stevenson, A. J. King, and S. Thirdborough, unpublished observations). Our data using the known CEA-derived peptide sequence indicate that the p.DOM-peptide design may be applicable to other cancer Ags. Using this model, it was also possible to demonstrate the advantage of removing the second domain of FrC, because potentially competitive epitopes within that domain were able to depress induction of CEA-specific CTLs. This finding confirms the principle behind the design and points toward relevance for human vaccines. Clearly, this design needs to be tested in cancer models in which protective immunity can be assessed, and it will be of interest first to assess protection against an EL4-CEA transfectant. The fact that our first candidate peptide from CEA generates a rapid and high level of CTLs from this format is encouraging.

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