Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization

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Abstract

We examined the role of the cellular localization of antigen on the immune response after DNA immunization of mice with three forms of ovalbumin (OVA). DNA encoding OVA which was secreted (sOVA) generated 10- to 100-fold higher IgG responses with 50- and 100-fold higher levels of IgG1 than the cytoplasmic (cOVA) or membrane bound (mOVA) forms. An IgG2a predominance was seen only in cOVA and mOVA immunized mice. Although the antibody response was CD4+ T cell dependent, the differences in the antibody response could not be compensated for by provision of excess CD4+ T cell help in TCR transgenic mice. Together with our hapten-carrier studies, this would indicate that membrane or intracellular localization limits the availability of antigen for B cell priming which affects the magnitude and form of the antibody response. Surprisingly, stronger cytotoxic T lymphocyte (CTL) responses were generated for sOVA or mOVA than for cOVA via intramuscular (i.m.) injection. Since a cytoplasmic antigen should have best access to the canonical class I pathway for antigen presentation, our results indicate that priming of CTL responses after i.m. DNA immunization is probably by cross-presentation of antigen by non-transfected professional antigen-presenting cells. In contrast, intradermal immunization with cOVA produced optimal CTL responses but, as with mOVA, suboptimal antibody responses. This, together with our ex vivo RT-PCR analysis showing similar mRNA levels from all three constructs 7 days post-immunization, argues against the differential CTL response for i.m. injection to be due to dose.

Introduction

Delivery of mammalian expression plasmids by direct intramuscular (i.m.) injection (1) or with biolistic systems (2) results in the uptake of the DNA and expression of the encoded protein. Injection of DNA i.m. usually results in a low level of protein expression due, at least in part, to the small percentage of myocytes transfected (1) and this has restricted the application of DNA injection in gene replacement therapies (3). However, DNA injection has been used to induce both humoral and cellular immune responses to encoded antigens (4) and these responses can persist for >1 year (5). The ability to generate a cytotoxic immune response with DNA immunization makes it auspicious for vaccination against intracellular pathogens, and indeed DNA vaccines have been shown to protect mice from challenge with viruses (4) and parasites (6,7 and see review 8).

The mechanisms underlying the induction of immune responses after DNA immunization are unclear. Since myocytes express MHC class I at low levels and do not
constitutively express class II or co-stimulatory molecules such as B7 (9), they appear unlikely candidates for the induction of antibody or cytotoxic T lymphocyte (CTL) responses. It is possible that low level transfection of antigen-presenting cells (APC) occurs at the injection site and these APC then traffic to lymphoid organs and present the encoded antigen to B and T cells (10) as has been shown after intradermal (i.d.) (11) and biolistic DNA immunization (12). Alternatively the myocyte may act merely as a source of antigen and priming occurs in the draining lymph node. In the latter case, optimum immune induction would result if the antigen was released from the myocyte by secretion or subsequent to cell damage.

Also unclear is the importance of the antigen’s cellular localization on the immune response after DNA immunization. Immunization of mice with plasmid DNA encoding the nucleoprotein of the lymphocytic choriomeningitis virus i.m. (13,14) generated poor immune responses and incomplete protection from challenge. The intracellular localization of this antigen may have had an influence on the poor responses. However, the DNA encoding influenza nucleoprotein induced quite strong antibody and CTL responses which confer a high degree of protection (4). To investigate the effect of the cellular localization of antigen, we constructed three forms of OVA, a secreted, membrane bound and cytoplasmic form, and compared how each induced antibody and CTL responses after i.m. DNA immunization.

Methods

Mice

Female mice aged 6–8 weeks were used in all experiments. Mice were maintained in specific pathogen-free conditions. Female mice aged 6–8 weeks were used in all experiments.

Plasmids and immunizations

Three forms of chicken OVA were constructed under the control of the CMV promoter in the mammalian expression plasmid pCI (Promega, Madison, WI). The full length OVA cDNA was obtained from Dr F. Carbone (Monash University, Melbourne, Australia) and an EcoRI–XbaI fragment was subcloned into pCI to yield the naturally secreted product (sOVA). An expression plasmid encoding a cytoplasmic form of OVA (cOVA) was constructed by deletion of the coding sequence for amino acids 20–145 of sOVA with SacI as described by Tabe et al. (19). The cDNA for a membrane localized OVA (mOVA; Dr Paul Gleeson, Monash University, Melbourne, Australia) encoding for the first 118 amino acids of the transferrin receptor linked to amino acids 138–385 of OVA, was subcloned into pCI via EcoRI–XbaI restriction digestion. Plasmids for injection were prepared from Escherichia coli by PEG precipitation as described (20) except that volumes of Solutions I, II and III were adjusted such that pellets were resuspended in 50 ml of Solution I for each liter of broth media used. Endotoxin was removed from plasmid preparations by four Triton X-114 phase separations (21) and DNA was stored at –20°C in normal saline until injected. The resultant plasmid preparations contained <10 IU endotoxin/mg of plasmid DNA as determined by the limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD). Mice received 100 µg of plasmid DNA in both quadriceps or i.d. at the base of the tail on day 0 and 14 of each experiment.

Antibody assays

Microtiter plates (Dynatech, Chantilly, VA) were coated with OVA protein (A-5503; Sigma, St Louis, MO; 10 mg/ml in PBS) by overnight incubation at 4°C and washed 4 times with PBS to remove unbound antigen. Plates were incubated with serially diluted sera in blocking buffer and washed 5 times with PBS to remove unbound antibody, plates were incubated with peroxidase conjugated anti-mouse IgG, IgG1, IgG2a or IgG2b antibodies (Southern Biotechnology, Birmingham, AL) diluted in blocking buffer. After washing 5 times with PBS, the amount of bound antibody was determined by addition of substrate solution [0.1 mg/ml 3,3,5,5-tetramethylbenzidine (T2885; Sigma) 0.03% H2O2 in 0.1 M sodium acetate, pH 6.0]. The reaction was stopped with 1 M H2SO4 and the OD read at 450 nm. Titers were defined as the highest dilution to reach an OD of 0.2.

To calibrate the IgG subclass ELISA, plates were coated with IgG1, IgG2a or IgG2b from mouse myelomas (10 mg/ml in 0.5 times PBS) overnight at 4°C, washed 3 times with PBS and then incubated with serially diluted anti-mouse IgG subclass horseradish peroxidase (HRP)-conjugated antibody. The dilution of each anti-mouse subclass antibody which gave identical absorbances in the ELISA were used subsequently.

The nitrophenol (NP)-specific response was determined from mice which received 500 µg of soluble NP conjugated to OVA (NP-OVA) by ELISA as above except that plates were coated with NP conjugated to BSA at molar ratios 13:1 (NP-13) for low affinity detection (22) (all NP reagents were kindly supplied by Dr David Tarlinton, Walter and Eliza Hall Institute).

CTL assays

Splenocytes from mice were stimulated with irradiated (200 Gy) E.G7-OVA cells (EG7) or irradiated (15 Gy) spleen cells osmotically loaded with OVA protein (23) for 5 days in 30 ml of tissue culture media (RPMI with 10% FCS). After washing with media, cells were counted and serially diluted in 96-well microtiter plates. A standard 5 h31Cr-release assay was performed with 1×104 EL4 or EG7 cells as targets at various effector to target ratios. The percent specific lysis was calculated as: [(c.p.m. of sample – c.p.m. of spontaneous release)/(c.p.m. of maximum release – c.p.m. of spontaneous release)]×100. Spontaneous release was defined as the mean c.p.m. released from five replicates of 1×104 labeled cells incubated in media alone. Maximum release was defined as the mean c.p.m. released from five replicates of 1×104 labeled cells incubated in media containing Triton X-100.
RT-PCR
Mice were injected i.m. with 100 µg of sOVA, cOVA or mOVA and the entire quadriceps removed 7 days later. RNA was prepared from muscle tissue using RNeazol B according to the manufacturer’s instructions (Biotecc, Houston, TX). PCR was performed on cDNA samples after reverse transcription of 1 µg of RNA. A 500 bp OVA amplicon was generated using the oligonucleotides OVA488f (5’-CTGAGCTAGCCAGAGGCTCAATTCC) and OVA931r (5’-CGGGATCCATCTTCATAAGTTAAGGTA) and a control 530 bp actin amplicon (GTGGGCCGCCCTAGGCA). Ten-fold serial dilutions of cDNA were used as templates for PCR using 25 cycles, to obtain linear amplification, of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s.

EL4 transfection
EL4 cells were transfected with 20 µg of sOVA, cOVA or mOVA and 1 µg pGKNeo, to allow drug selection, by electroporation at 0.25 V and 960 µF in a Gene Pulsar II (BioRad, Richmond, VA). Transfectants were selected and maintained in media containing 0.5 mg/ml Geneticin (Gibco/BRL, Gaithersburg, MD).

OVA expression in EL4 cells was examined after bio-synthetic labeling by centrifugation for 5 h in 1 ml of methionine-free RPMI containing 200 µCi [35S]methionine. Supernatants were collected and the cells washed 3 times with ice-cold PBS and then lysed in 100 µl of lysis buffer (10 mM CHAPS, 50 mM HEPES, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotonin, pH 7.4) for 30 min at 4°C. After clarification by centrifugation (100,000 g for 30 min at 4°C), lysates and supernatants were pre-cleared by incubation with 10 µl of a 1:1 slurry of Protein A–Sepharose (Pharmacia, Uppsala, Sweden) in PBS, to which 5 µl of normal mouse serum had been prebound. Supernatants were immunoprecipitated by incubation overnight at 4°C with 10 µl of Protein A–Sepharose to which 5 µl of mouse anti-OVA serum had been prebound. Immunoprecipitates were then washed once in high-salt buffer (0.5% Triton X-100, 500 mM NaCl, 50 mM NaH2PO4), twice in PBS plus 0.5% Triton X-100 and once in Tris–glycine buffer (0.5% Triton X-100, 14.7 mM Tris, 192 mM glycine, pH 7.4). Immune complexes were eluted from the beads by boiling for 3 min in 20 µl double-strength sample buffer and resolved by 10% SDS–PAGE. Gels were dried and exposed on a phosphor-imager (Molecular Dynamics, Sunnyvale, CA).

To examine expression of OVA on the cell surface, 1 × 10^6 cells were incubated with rabbit anti-OVA sera for 30 min on ice and then washed 3 times with FACS buffer. Bound Ig was detected with FITC-conjugated anti-rabbit Ig (Silenus, Miami, FL) by incubation for 30 min on ice. After washing 3 times with FACS buffer samples were analyzed with a FACScan and Lysys II software (Becton Dickinson, San Francisco, CA).

Proliferation assay
Splenocytes from OT-I mice were used to test the antigen-presenting capacity of the EL4 transfectants. EL4 and EL4-OVA transfectants were treated with mitomycin C (50 µg/ml; Sigma) and irradiated (60 Gy), and were used as stimulators.

Briefly, OT-I splenocytes were incubated for 3 days at 37°C in 96-well round-bottom tissue culture plates with EL4 or EL4 transfecant cell lines. The OVA expression in 1 × 10^7 cells was examined after bio-synthetic labeling by centrifugation for 5 h in 1 ml of methionine-free RPMI containing 200 µCi [35S]methionine. Supernatants were collected and the cells washed 3 times with ice-cold PBS and then lysed in 100 µl of lysis buffer (10 mM CHAPS, 50 mM HEPES, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotonin, pH 7.4) for 30 min at 4°C. After clarification by centrifugation (100,000 g for 30 min at 4°C), lysates and supernatants were pre-cleared by incubation with 10 µl of a 1:1 slurry of Protein A–Sepharose (Pharmacia, Uppsala, Sweden) in PBS, to which 5 µl of normal mouse serum had been prebound. Supernatants were immunoprecipitated by incubation overnight at 4°C with 10 µl of Protein A–Sepharose to which 5 µl of mouse anti-OVA serum had been prebound. Immunoprecipitates were then washed once in high-salt buffer (0.5% Triton X-100, 500 mM NaCl, 50 mM NaH2PO4), twice in PBS plus 0.5% Triton X-100 and once in Tris–glycine buffer (0.5% Triton X-100, 14.7 mM Tris, 192 mM glycine, pH 7.4). Immune complexes were eluted from the beads by boiling for 3 min in 20 µl double-strength sample buffer and resolved by 10% SDS–PAGE. Gels were dried and exposed on a phosphor-imager (Molecular Dynamics, Sunnyvale, CA).

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Results
Effect of cellular localization on the antibody response
In preliminary experiments, we had confirmed the localization of the various forms of OVA in EL4 transfectants. By immuno-precipitation, OVA was found in the supernatant of [35S]Met-biosynthetic labeling of the indicated cell lines. (B) FACS analysis of membrane OVA expression on EL4-mOVA transfectants when primary (rabbit anti-OVA) antibody was included (solid line) or excluded (dashed line). No surface expression of OVA could be detected on EL4 control cells or cells transfected with cOVA or sOVA (data not shown).

Effect of cellular localization on the antibody response
In preliminary experiments, we had confirmed the localization of the various forms of OVA in EL4 transfectants. By immuno-precipitation, OVA was found in the supernatant of [35S]Met-labeled EL4-sOVA cells (Fig. 1A); to a lower extent also in the supernatant of EL4-mOVA transfectants presumably by proteolytic cleavage from cell surface. For EL4-cOVA cells, OVA was found in lysates but not in the supernatant (Fig. 1A); cytoplasmic localization of this construct has also previously been shown in oocytes (19). Flow cytometry showed OVA on the surface of only EL4-mOVA cells (Fig. 1B).

BALB/c mice (H-2^d haplotype) were immunized i.m. with 100 µg of sOVA, cOVA or mOVA in both quadriceps on day 0 and 14. The OVA specific IgG response was measured by ELISA at 2, 4 and 8 weeks post initial (day 0) injection. In numerous experiments, mice receiving vector or saline did not mount an OVA-specific immune response. The sOVA immunized mice had peak IgG titers (expressed as log_{10}
Fig. 2. OVA-specific IgG responses in DNA immunized mice. Sera were obtained from BALB/c or CBAxC57Bl/6 mice immunized with sOVA, cOVA or mOVA at the indicated times post-immunization and stored at –20°C until assayed for OVA-specific IgG in an ELISA. Titers were defined as the highest dilution to give a 0.2 OD at 450 nm. Results are expressed as the mean of the log_{10} titer ± SEM from five mice in each group.

The OVA-specific IgG response was examined in BALB/c mice at 4 and 8 weeks post-immunization. The sOVA immunized mice had 50- and 100-fold higher IgG1 titers than cOVA and mOVA immunized mice respectively at 4 weeks, and at least 10-fold higher titers at 8 weeks (Fig. 3, P < 0.05). This increase in IgG1 would probably account for the difference seen in total IgG titers although higher IgG2a titers were obtained in sOVA immunized mice compared to cOVA immunized mice (P < 0.05). The IgG2a and IgG2b titers were similar between sOVA and mOVA immunized mice at all time points. Similar results were obtained in CBAxC57Bl/6 mice (data not shown).

There was an apparent difference in the subclass predominance in mOVA immunized mice due to the low IgG1 response. To illustrate the subclass dominance in the response of individual mice we compared the ratios of IgG1 log_{10} titer to IgG2a log_{10} titer (Table 1). At 4 weeks the mice immunized with sOVA had higher IgG1/IgG2a ratios than cOVA and mOVA immunized mice (P < 0.05) due to an IgG2a dominance in the cOVA and mOVA immunized mice. These differences were still significant (P < 0.05) at 8 weeks although less pronounced. Interestingly, OVA-specific IgM, IgG3 or IgE responses could not be detected above background after DNA immunization (data not shown).

Antibody responses in OT-I, CD4-deficient and CD8-deficient mice
Mice genetically deficient for CD4 were used to determine the role of CD4^+ T cells in the generation of the OVA antibody
response. The antibody response is completely ablated in the absence of CD4+ T cells (Fig. 4A). This was expected as these mice do not mount an antibody response after immunization with OVA protein in complete Freund's adjuvant (data not shown). Moreover, OT-I mice which have a severe deficiency of CD4+ T cells but are transgenic for OVA-specific CD8+ T cells did not generate an antibody response to OVA after DNA immunization (Fig. 4A). On the other hand, CD8-deficient mice were capable of generating an antibody response. Indeed, the absence of CD8+ T cells results in an enhanced antibody response at 4 weeks post-immunization (Fig. 4B, P < 0.05) even though CD4+ T cell numbers are similar to the wild-type controls (24). Together these results confirm the essential role of CD4+ T cells in the generation of antibody responses after DNA immunization.

**Antibody responses in DO mice**

We felt that localization of antigen could affect the availability of antigen for presentation to both B and the CD4+ Th cells necessary for the generation of an antibody response. We investigated whether the differences in the antibody responses might be due to the relative priming of OVA-specific CD4+ Th cells. We therefore examined the responses generated in TCR transgenic DO mice (BALB/c background) which have large numbers of such cells recognizing OVA323–339 in the context of I-A^d (16). Surprisingly, the IgG responses of these TCR transgenic mice 4 weeks after immunization with the OVA plasmids were similar to those in normal BALB/c mice (pre-boost in Table 2 compared to Fig. 2A). These mice were then boosted with NP-OVA to determine if in vivo OVA-primed T cells from DNA immunizations (as carrier) would affect the response to another B cell determinant (the hapten NP) and to assess the secondary B cell response to soluble OVA in DNA immunized mice. NP antibody responses were increased when mice were primed by DNA immunization (Table 2). However, there was no difference in the NP response among mice immunized with the three forms of OVA. This would suggest that carrier priming of T cells by the three forms of OVA had been similar. In contrast to the anti-hapten responses, anti-OVA antibody responses were only boosted in the sOVA immunized mice (38-fold). Anti-OVA antibody responses in mOVA and cOVA immunized mice remained low or undetectable. This would argue that the three forms of OVA primed Th cells similarly but the secreted form had primed B cells more efficaciously and therefore had produced the highest antibody response.

**Effect of cellular localization on CTL responses**

CBAxC57Bl/6 mice were injected i.m. at days 0 and 14 with the sOVA, cOVA or mOVA plasmids. At 6–8 weeks post initial immunization splenocytes were used to measure OVA-specific CTL activity, using EG7 cells as OVA targets and EL4 cells as control targets. The OVA-specific CTL responses in sOVA and mOVA immunized mice were similar and higher than the response in cOVA immunized mice (Fig. 5, P < 0.05, t-test paired for all four effector:target ratios). Results shown are pooled from two independent experiments. As expected, splenocytes from control animals were not able to lyse EG7 cells and splenocytes from immunized mice did not lyse the EL4 control cells (data not shown).

The reduced CTL response in cOVA immunized mice may have been due to an impaired ability of the cOVA product to be presented as OVA257–264, the major Kb-restricted CTL epitope of OVA (25), and induce an optimal CTL response. Indeed, the absence of CD8+ T cells results in an enhanced antibody response at 4 weeks post-immunization (Fig. 4B, P < 0.05) even though CD4+ T cell numbers are similar to the wild-type controls (24). Together these results confirm the essential role of CD4+ T cells in the generation of antibody responses after DNA immunization.

**Table 1. Cellular localization of antigen influences the IgG subclass predominance in DNA immunization**

<table>
<thead>
<tr>
<th>Immunogenb</th>
<th>Ratio IgG1:IgG2a</th>
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<tbody>
<tr>
<td>sOVA</td>
<td>1.28 ± 0.29</td>
</tr>
<tr>
<td>cOVA</td>
<td>0.72 ± 0.31</td>
</tr>
<tr>
<td>mOVA</td>
<td>0.36 ± 0.02</td>
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<table>
<thead>
<tr>
<th></th>
<th>4 weeks</th>
<th>8 weeks</th>
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<tbody>
<tr>
<td>sOVA</td>
<td>1.17 ± 0.26</td>
<td>1.16 ± 0.24</td>
</tr>
<tr>
<td>cOVA</td>
<td>0.81 ± 0.21</td>
<td>0.81 ± 0.22</td>
</tr>
<tr>
<td>mOVA</td>
<td>0.69 ± 0.27</td>
<td>0.68 ± 0.27</td>
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*aThe ratio of IgG1 to IgG2a titers were determined by the log_{10} of IgG1 divided by the log_{10} titer of IgG2a.*

*bMice were immunized i.m. with 100 µg of plasmid DNA in both quadriceps on day 0 and 14.
Localization of antigen affects DNA immunization

Table 2. B cell priming is a limiting factor in DNA immunization of DO mice

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Mean log_{10} IgG titer</th>
<th>Pre-boost^d</th>
<th>Post-boost</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NP-13^c</td>
<td>ND^e</td>
<td>ND</td>
</tr>
<tr>
<td>sOVA</td>
<td>NP-OVA</td>
<td>1.57 ± 0.36</td>
<td>ND^e</td>
</tr>
<tr>
<td>mOVA</td>
<td>NP-OVA</td>
<td>1.94 ± 0.13</td>
<td>1.44 ± 0.49</td>
</tr>
<tr>
<td>cOVA</td>
<td>NP-OVA</td>
<td>2.06 ± 0.17</td>
<td>4.12 ± 0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.54 ± 0.31</td>
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</tbody>
</table>

^aMice were immunized i.m. with 100 µg of plasmid DNA in both quadriceps on day 0 and 14.
^bMice were boosted i.p. with 500 µg of soluble NP-OVA on day 28.
^cAge-matched DO female mice were bled 2 weeks post boosting and the NP IgG response determined by ELISA using NP-13 as antigen.
^dTiters were determined as the highest dilution to reach an OD of 0.2 and the mean of the log_{10} titer ± SEM for each group is shown.
^eND, not detectable

Fig. 5. CTL responses in i.m. DNA immunized mice. Splenocytes from CBA×C57Bl/6 mice were taken 6 weeks post-immunization with sOVA, mOVA and cOVA, re-stimulated in vitro for 5 days, and tested for their ability to lyse EG7 cells in a standard 51Cr-release assay. Results shown are the mean specific lysis from four mice ± SEM in each immunized group and an un-immunized age-matched mouse as control.

Fig. 6. Proliferation of OT-I splenocytes is limited by the number of EL4 transfectants (as APC) added. EL4 cells transfected with the indicated plasmids were treated with mitomycin C (50 µg/ml), irradiated (50 Gy) and used as stimulators for 5×10^4 OT-I splenocytes in a standard 3 day proliferation assay. Background proliferation of EL4 and EL4 transfectants is shown as an open bar and proliferation of OT-I cells in response to titrated stimulators is shown as a solid bar. OT-I T cells alone did not show significant proliferation. Results are expressed as the mean c.p.m. ± SD from three replicate wells.

was seen when incubated alone or when control EL4 cells were added.

Differences in immune responses not due to dose

The reduction of CTL and antibody responses for cOVA could have been due to lower expression levels. Unfortunately we could not address this directly by examination of protein levels in vivo because they were below the detection limits of Western blotting and immunohistochemistry (data not shown). We therefore chose to examine mRNA levels in vivo to provide an indication of expression levels in immunized mice. A luciferase reporter construct was used to study protein expression in vivo. Luciferase expression peaked 7–14 days post plasmid injection but could still be detected up to 28 days post-injection (data not shown). Therefore for our semi-quantitative mRNA studies, muscles were removed from mice 7 days post DNA injection and from un.injected mice as control. Since the OVA amplicon did not span any intronic sequences, RT-negative samples were used to control for amplification resulting from plasmid DNA contamination. No bands of the expected size for the OVA amplicon could be detected when reverse transcriptase was omitted, whereas bands of similar intensity were seen in PCR from two different muscle samples from mice immunized with the three OVA DNA constructs (Fig. 7). The OVA amplicon could not be detected at any further dilutions of the cDNA (up to 1:100, data not shown) whereas an actin amplicon could be detected at further
Localization of antigen affects DNA immunization

Fig. 7. RT-PCR analysis of OVA mRNA in DNA immunized mice. Semi-quantitative RT-PCR was used to amplify an OVA-specific product or actin control on RNA samples prepared from quadriceps muscles from mice injected with plasmid DNA or an un-immunized control.

dilutions and the 1:10 dilution is shown (Fig. 7). The results are consistent with the view that all three forms of OVA were expressed in muscles at similar levels at least in terms of RNA expression.

Further evidence that the lower CTL response of cOVA was due to antigen localization and not dose was obtained when mice were immunized via the i.d. route. It has been shown that i.d. immunization results in the transfection of APC (11) and if this were the case, antigen localization should have less effect on the induction of a CTL response. Indeed, mice immunized with cOVA via the i.d. route had quite strong OVA-specific CTL responses that were now similar to those from sOVA and mOVA immunized mice (Fig. 8A). The similarity of CTL responses with the three forms of OVA after i.d. immunization supports the premise that there is not significant dose variation among the three plasmid immunizations. As controls, splenocytes from naive animals were not able to lyse EG7 cells and splenocytes from immunized mice did not lyse the EL4 control cells (data not shown). However, the differences in the antibody responses seen after i.m. immunization were also seen after i.d. immunization (Fig. 8B). Expression of OVA in the cytoplasm of APC after direct transfection during i.d. immunization with cOVA would not be expected to prime B cells (as antigen would not be accessible to surface Ig of B cells). This is consistent with the above hapten-carrier studies which suggested that altering the localization of antigen affected the ability to prime B cells and therefore the antibody response.

Fig. 8. Immune responses in i.d. DNA immunized mice. (A) CTL responses. Splenocytes from CBA×C57Bl/6 mice were taken 6 weeks post-immunization with sOVA, mOVA and cOVA, re-stimulated in vitro for 5 days, and tested for their ability to lyse EG7 cells in a standard 51Cr-release assay. Results shown are the mean specific lysis ± SEM for two mice in each immunized group and an un-immunized age-matched control. (B) Antibody responses. Sera were obtained from CBA×C57Bl/6 mice immunized with sOVA, cOVA or mOVA at 6 weeks post-immunization and stored at –20°C until assayed for OVA-specific IgG in an ELISA. Titers were defined as the highest dilution to give a 0.2 OD at 450 nm. Results are expressed as the mean of the log10 titer ± SEM from five mice in each group.

Discussion

Our results show that the cellular localization of the antigen influences both the cellular and the humoral immune response after DNA immunization. A secreted OVA induced higher IgG responses than cytoplasmic or membrane bound OVA in all strains tested. Although the antibody response was shown to be CD4+ T cell dependent, a plausible explanation of why secreted OVA was better at inducing antibody responses is that the differences observed were due to antigen availability for priming of B cells, since provision of excess CD4+ T cell help in a transgenic model did not alter the response. Both the membrane bound and secreted OVA induced high CTL responses whereas the CTL response to the cytoplasmic form was low. There are several lines of evidence indicating that the differences in the antibody and CTL responses elicited by the various forms of OVA were not due to dose of protein produced. The three forms of OVA were made with identical plasmid backbone and produced similar RNA levels in vivo. Furthermore the antibody response but not the CTL response was affected by membrane localization. Finally, the CTL responses upon i.d. immunization with cOVA, mOVA and sOVA were similar. These results should be of importance in the design of DNA vaccines and provide insight into the mechanism of immune induction after DNA immunization.

In preliminary experiments we had immunized mice with plasmids expressing luciferase and were unable to detect antibody directed against luciferase despite readily detecting expression of this reporter in the muscles of immunized mice. Since mice immunized with luciferase protein did mount an antibody response, we surmised that the intracellular
Localization of luciferase within peroxisomes (26) might be responsible for the lack of antibody response to luciferase-encoded DNA immunized mice (data not shown). As mentioned earlier, mice immunized with DNA encoding other intracellular proteins, e.g., the nucleoprotein of the lymphocytic choriomeningitis virus, also do not mount strong immune responses (13,14). The one exception is the influenza nucleoprotein (4) but that may be because it is in part secreted (27) and this may account for its increased immunogenicity. Xiang et al. (28) examined the immune response after DNA immunization with plasmids encoding a secreted or membrane bound form of rabies glycoprotein and found a slight increase in the antibody response in mice receiving the secreted form and no difference in the CTL response, which is in agreement with our results. If the induction of OVA-specific antibody occurs in the draining lymph node, the reason why sOVA was optimum at inducing antibody responses might be that a secreted antigen would be more efficient than a membrane bound or cytoplasmic antigen at reaching the lymph node. Our hapten-carrier studies and the fact that excess of T<sub>H</sub> precursors do not augment antibody responses indicated that the restriction of antigen availability for B cell priming may be the limiting factor. Therefore, a cytoplasmic or membrane localization would be less efficient than a secreted one at achieving this and thus had several effects on the antibody response. The total IgG response was at least 10-fold lower than that of the secreted antigen, or delayed in the case of membrane bound antigen, primarily due to lower IgG1 induction. For mOVA, antigen would only be available to B cells and APC in the draining lymph node after it was proteolytically cleaved and released from the cell membrane, thereby delaying the response. Interestingly, Hodgkin et al. (29) found that up to six divisions were required for isotype switching to IgG1 during B cell differentiation and continuous presence of antigen is required for these divisions. Furthermore, provision of excess T<sub>H</sub> cell signal did not alter the level of IgG1 expression nor the proportion of B cells expressing IgG1. This suggested that B cell differentiation and IgG1 expression is the result of stochastic and predetermined events during consecutive B cell divisions. Consistent with the notion that antigen presentation by professional APC may be limiting in DNA immunization is the observation that co-injection of a plasmid expressing granulocyte macrophage colony stimulating factor, which is a potent activator of APC, enhanced the antibody response to rabies antigen (30). However, our conclusion is inconsistent with a report that injection of a plasmid expressing human carcinoembryonic antigen and the co-stimulatory molecule B7-1 enhanced antibody responses, albeit not greatly (31). If B7-1 acts to stimulate T<sub>H</sub> cells via CD28, it remains questionable how myocytes could stimulate the TCR of T<sub>H</sub> cells in the absence of class II. Furthermore, we have shown directly that even provision of vast excess of T<sub>H</sub> in a TCR transgenic model did not affect the antibody response. This would suggest that even enhanced presentation of antigen to T cells by a non-professional APC should not alter the antibody response.

The IgG subclass ratio was altered to an IgG2a dominance in the case of cOVA and mOVA immunizations. DNA immunization resulting in an IgG2a dominance has also been reported for herpes simplex virus glycoprotein B (32) and Plasmodium yoelii circumsporozite protein (33) both of which are membrane bound antigens. The similarity in the response after DNA immunization and viral infection, including an IgG2a dominance, has been reported (34). DNA immunization would mimic a viral infection in that small amounts of endogenously produced antigen are continuously available but differs in the non-infectious and non-lytic nature of DNA immunization. The immunology of virus infections may therefore be applicable to DNA immunization. Zinkernagel (35) highlighted the importance of the viral antigen as a driving force in the immune response. Our results also indicate that the antigen itself is very important in determining the form and magnitude of the antibody response. We argue that antigen availability for B cell priming in the lymphatic tissue is critical in DNA immunization and that by rendering the antigen membrane bound or cytoplasmic, this availability as a key driving force is decreased. Thus, the overall IgG response is not only lower but shifted to an IgG2a dominance (or decreased IgG1) which is in agreement with the model discussed by Thompson (36). In this model, the dose of antigen acts to drive the immune response from a low dose, T<sub>H</sub><sub>1</sub> (IgG2a) default to a high dose, T<sub>H</sub><sub>2</sub> (IgG1) dominated response. By our arguments, the effective dose of a secreted antigen would be higher because it can reach B cells more easily and hence there is a substantial IgG1 response after sOVA immunization. Injection of soluble OVA protein which is thus not cell associated and hence more closely mimics sOVA immunization also produces a dominant IgG1 response (data not shown).

Another factor not examined directly in this study which may have affected the antibody responses in sOVA, mOVA and cOVA immunized mice is the number of immunostimulatory sequences (CpG motifs) in the plasmid DNA itself. Interestingly, it has recently been shown that DNA containing these specific sequences is able to stimulate cells to augment the CTL and antibody response to the cytoplasmic antigen β-galactosidase after i.d. DNA immunization (37). The sequence AACGTT was particularly potent in that only one site was required to enhance the cellular and humoral response to β-galactosidase but having two sites conferred no advantage. All of the plasmids (based on pCI) we used in this study have at least three such sites: sOVA.pCI has four, cOVA.pCI and mOVA.pCI have three. It would be possible, but unlikely, that reduction from four to three sites could explain the reduced CTL response to cOVA after i.m. immunization given that a reduction from two sites to one site made no difference in the response to β-galactosidase discussed above. In any case, this could not explain the difference in the CTL response between cOVA and mOVA immunized mice.

In DNA immunization, the cellular localization of antigen could influence the priming of CTL response if it enables the antigen better access to the class I processing pathway. There are several lines of evidence that would suggest an antigen localized to the cytoplasm would be optimum for inducing a CTL response. The majority of CTL epitopes originate from proteins expressed in the cytosol and the subsequent presentation of processed peptides (38). Cytoplasmic expression of protein fragments is all that is required for a cell to act as a target for CTL (39). Loading of soluble protein into the cytosol is an efficient method for inducing CTL (23). Our in vitro results showed that EL4 cells transfected
with cOVA as APC were capable of presenting OVA257–264 to OT-1 T cells which proliferated as did those stimulated with mOVA or sOVA EL4 transfectants. However, in the case of i.m. but not i.d. DNA immunizations, we found that the cytoplasmic localization of OVA resulted in a lower CTL response compared with the secreted or membrane bound forms. This would indicate that the cell(s) transfected by i.m. DNA immunization in vivo are inefficient at priming a CTL response themselves and would therefore argue against transfection of professional APC playing a major role in CTL induction, as is postulated for i.d. (11) and biolistic (12) DNA immunizations.

Recently, the mechanism underlying the generation of CTL after DNA immunization with plasmids encoding human immunodeficiency or herpes simplex virus antigens was investigated (40). It was shown that scid mice of the H-2b or H-2d haplotype infused with b x d F1 spleenocytes generated CTL against the DNA encoded antigen which was restricted by the scid host haplotype. However, if these mice also received b x d bone marrow then CTL of both b and d haplotypes were generated indicating that this cross-priming was dependent upon presentation of antigen by a bone marrow-derived cell. Of relevance is the findings of Norbury et al. (41) who showed that bone marrow-derived dendritic cells were capable of TAP-dependent presentation of soluble antigen which occurs after macrophagocytosis by membrane ruffling activity. Furthermore, bone marrow-derived cells exposed to exogenous OVA in vivo have been shown to prime a CTL response (42). A cross-presentation mechanism supports our supposition that the cell(s) transfected by i.m. DNA immunization, most likely the myocyte, does not directly prime the CTL response. Our results would indicate that cytoplasmic OVA is not transferred to the bone marrow-derived APC as efficiently as the membrane bound or secreted form and therefore serves to limit the number of effective APC.

The use of DNA immunization as a vaccine shows enormous promise for many reasons including facility to modify the antigen, economy, facility to produce large amounts and the native state of the protein produced. We have shown the importance of the cellular localization of the antigen on the immune response which may be directly applicable to improving currently investigated DNA vaccines. Our results are of relevance to understanding the mechanism of immune induction after DNA immunization and provide further evidence that the myocyte be considered only as a source of antigen and that priming of CTL and B cells which probably occurs in the draining lymph node.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>cOVA</td>
<td>cytoplasmic OVA</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>DO</td>
<td>OVA-I-A^* specific CD4^+ TCR transgenic mice</td>
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<tr>
<td>i.d.</td>
<td>intradermal</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
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<td>mOVA</td>
<td>membrane bound OVA</td>
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<tr>
<td>NP</td>
<td>nitrophorin</td>
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<td>NP-OVA</td>
<td>nitrophorin conjugated to OVA</td>
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<td>OT-1</td>
<td>OVA-K^b-specific CD8^+ TCR transgenic mice</td>
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<tr>
<td>sOVA</td>
<td>secreted OVA</td>
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References

CD8 is needed for development of cytotoxic T cells but not helper T cells. Cell 65:443.


