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In Vivo Priming by DNA Injection Occurs Predominantly by Antigen Transfer[1]

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DNA vaccines can stimulate both humoral and cytolytic immune responses. Although bone marrow-derived elements present the expressed Ag, the mechanisms for acquiring immunogenic peptides have yet to be fully elucidated. APCs may become directly transfected by plasmid DNA or process extracellular proteins produced by other transfected cells. Using a transactivating plasmid system and bone marrow chimeras, we show that both mechanisms appear to be involved; however, the bulk of the immune response is dependent on expression of Ag by nonlymphoid tissues and transfer to APCs. These in vivo studies are the first to define the role of transfected nonlymphoid cells in generating Ag for presentation by bone marrow-derived APCs after needle injection with plasmid DNA. *The Journal of Immunology,* **1999, 163: 4721–4727.**

Potent and long lived immune responses have been demonstrated after the injection of naked plasmid DNA into the dermis or muscle tissue of mice (1, 2). Consequently, gene vaccination is being evaluated for potential applications in the fields of infectious diseases, allergy, and cancer. Despite the avid interest in this method of immunization, the mechanisms of priming these humoral and cellular responses have yet to be fully elucidated. The cells that have been described to express the encoded Ag are predominantly found at the site of plasmid inoculation. However, studies using bone marrow chimeras showed that the antigenic peptide involved in priming a CTL response is presented in the context of the MHC-encoded class I molecule of bone marrow-derived cells and not by injected myocytes (3–5). Thus, immune responses are initiated by Ag that is acquired by APCs. These APCs may either become directly transfected themselves or process extracellular proteins.

Within the dermis, there are a variety of "professional" APCs such as Langerhans cells and macrophages that could be transfected by the injected plasmid DNA (6). Additionally, intradermal (i.d.)[3] gene vaccination has been shown to induce expression of the gene product in cells with macrophage and dendritic cell-like morphology as well as keratinocytes and dermal fibroblasts (2). A role for directly transfected Langerhans cells was suggested by the appearance of Ag-expressing dendritic cells in draining lymph nodes after biolistic immunization (7, 8). However, professional APCs are sparse in other tissues such as muscle, although they may be recruited by the local irritation that follows injection (9, 10). Hence, it would be rare that these APCs would become directly transfected by injected DNA.

In tissues that have few resident APCs, the transfected cells may act as an antigenic reservoir. Alternative mechanisms to present exogenous Ags in the context of MHC class I have been reported (11–16). Consequently, presentation may occur by protein transfer from transfected somatic cells to a professional APC. This potential mechanism is supported by the original cross-priming experiments in which bystander cells were shown to present MHC class I-restricted minor histocompatibility Ags in vivo (17) and in a more recent report in which a test Ag expressed by transfected myoblasts of one haplotype was able to cross-prime a CTL restricted to another MHC haplotype when transferred to F_1-recipient mice (18).

Several investigators have been able to isolate dendritic cells after DNA vaccination and demonstrate their ability to present Ag in vitro to hybridomas or cell lines (8, 19, 20). After our original observation that bone marrow-derived cells present plasmid encoded Ag to the immune system (4), we initially hypothesized that these directly transfected dendritic cells were largely responsible for priming the subsequent immune responses. In this paper, we present our surprising results that although directly transfected dendritic cells are potent APCs, the magnitude of the immune response is dependent on Ag produced by transfected nonlymphoid cells.

We designed parallel avenues of investigation to determine the relative contributions of Ag produced by connective tissue or by bone marrow-derived cells after naked plasmid DNA injection. First, we used a plasmid with a monocyte-specific promoter to determine whether directly transfected APCs of monocytic cell lineage were expressing immunogenic Ag (21). Poor humoral and CTL responses were elicited after injection with this plasmid, indicating that this attempt at targeting expression to the APC did not produce the usual response.

Second, to evaluate whether transfection of migratory cells or cells distant from the site of injection was necessary for immune recognition of antigenic protein, we used an expression suppressible plasmid system to separate transport of the plasmid vs protein from the site of injection (22, 23). Mice lacking B and T cells were immunized with and without the suppressing drug. Splenocytes (APCs) from these mice were injected into recipient mice and assessed for their ability to prime a CTL response. Recipient mice

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[3] Abbreviations used in this paper: i.d., intradermal; β-gal, β-galactosidase; tTA, tetracycline-controlled transactivating protein.

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that received splenocytes from mice without suppressive treatment mounted a greater CTL response, suggesting that exogenous transfer was important for the magnitude of the response. Additionally, a CTL response was not induced when the site of plasmid injection was ablated by amputation and the suppressing drug subsequently removed. The immunogenic protein was predominantly expressed locally at the site of injection and not in the draining lymph nodes or spleen.

Finally, we made chimeric mice that had either a transgenic transcriptional transactivator in their resident tissues or in their adoptively transferred bone marrow. Wild-type mice with transgenic bone marrow had weaker CTL and humoral responses than transgenic mice with wild-type bone marrow after injection of plasmid DNA that required the transactivator for high levels of expression. These data collectively indicated that nonlymphoid tissues predominantly expressed antigenic peptides encoded by plasmid DNA, which were then transferred to APCs to stimulate the bulk of the immune response.

Materials and Methods

Mice

OVA-tcr-1 mice were a kind gift from M. Bevan (University of Washington, Seattle, WA), and Rag-$1^{-/-}$, BALB/c, C57BL/6, and B6, C3-TgN(TettTALuc)1Dgs mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C3-TgN(TettTALuc)1Dgs mice express the tetracycline-controlled transactivating protein (tTA) and luciferase (24). OVA-tcr-1 mice are transgenic for a TCR that is specific for the H-$2K^b$-restricted epitope from OVA, SIINFEKL (25). The mice were bred and maintained under standard conditions in the University of California at San Diego Animal Facility, which is accredited by the American Association of Laboratory Care.

Plasmids

The construction of pACB and pCMVint-based vectors have been previously described (2). The vectors pUHD15-1, encoding the tTA, and pUHD10-3 were generous gifts of H. Bujard (Universität Heidelberg, Heidelberg, Germany) (22). The cDNA for hen egg OVA, a generous gift of N. Shastri (University of California, Berkeley, CA), was subcloned into pUHD10-3 (pUHD-OVA) and pACB (ACB-OVA). ACB-TPASIINFEKL was generated as described (26). A membrane-bound form of OVA fused to the transmembrane terminus of the transferrin receptor (TfROVA, a kind gift of R. Teasdale, Monash University, Melbourne, Australia) (27) was subcloned into the pCMVint vector. The pACB-Z plasmid expressed the *Escherichia coli* LacZ cDNA (28). The plasmid fXbaSpl-βgal included the *cis*-active elements responsible for transcriptional activation of the scavenger receptor and a β-galactosidase (β-gal) reporter gene (kind gift of C. Glass, University of California at San Diego) (21). Plasmid DNA for injection was prepared as previously described (4).

Transfer

Adult Rag-1-deficient mice had 15 mg (21 day) slow release tetracycline pellets (Innovative Research of America, Toledo, OH) s.c. implanted 1 day before injection. These mice were injected i.d. in the proximal tail with 50 μg each of pUHD-OVA and pUHD15-1 in 50 μl of normal saline twice, 3 day apart. One week after the first injection, the animals were sacrificed, and 1.3×10^7 splenocytes were transferred i.p. into OVA-tcr-1 mice. DNA was isolated from the pooled splenocytes and the proximal and distal portions of the tails as described (20). Detection of the pUHDOVA plasmid by PCR was performed using these primers 5′-TGCTGTTGCCTGAT GAAGTC-3′ and 5′-TACCACCTCTCTGCCTGCTT-3′. As a control for the integrity of the DNA primers for GADPH, the following sequences were used: 5′-TCTCATGGTTCACACCCATGACGAACATG-3′ and 5′-AAGAAGATGCGGCTGACTGTCGAGCCACAT-3′. PCR conditions were: 1 min at 95°C, 1 min at 50°C, and 1 min at 70°C for 30 cycles. The PCR products were analyzed by electrophoresis in 1.5% agarose gels. The detection limit of this PCR assay was 1 pg of plasmid DNA.

Ablation

Groups of adult C57BL/6 mice had 15 mg (21 day) slow release tetracycline pellets (Innovative Research of America) s.c. implanted 5 days before injection. These mice were injected i.d. in the distal tail with 50 μg each of pUHD-OVA and pUHD15-1 in 50 μl normal saline twice, 3 day apart. The wheal from injection did not extend to the proximal third of the tail. One week after the first injection, a suture was bound tightly around the tail base under metofane anesthesia and the tail was amputated near the rump with a razor blade. The tetracycline pellets were then removed. At week 2 the animals were sacrificed and their splenocytes harvested.

Bone marrow chimeras

Adult C57BL/6 and C3-TgN(TettTALuc)1Dgs were purchased from The Jackson Laboratory and bred in our colony. Mice 10–12 wk old were used as bone marrow recipients. Recipient mice were given 900 rads and then injected i.v. with 8×10^6 T-depleted bone marrow cells from young adult donors in a total volume of 100 μl of serum-free RPMI 1640 medium (BioWhittaker, Walkersville, MD) as previously described (4). After 6 wk, chimerism was verified by detecting the expression of luciferase in the peripheral blood by PCR for the luciferase gene and confirmed by assaying thymi for luciferase activity using Luciferase Assay System (Promega, Madison, WI) per the manufacturer's recommendations.

Cell transfections

In pilot experiments, each cell type was titrated for optimal cell density for highest transfection efficiency. In 6-well plates, 2.5×10^5 COS-1 cells, 5×10^5 for RAW-264.7 cells, and 2.5×10^6/well THP-1 were seeded. Adherent cells were incubated with 5 μg of plasmid in conjunction with 15 μl of SuperFect (Qiagen, Chatsworth, CA) in 5 ml of OptiMem1 (Life Technologies, Grand Island, NY) for 8 h. The transfection solution was then removed, and the cells were washed with PBS and refed with DMEM (BioWhittaker) 10% heat-inactivated FBS, 2 mM glutamine, 50 μM 2-ME, and 1% penicillin and streptomycin. The suspension cells were transfected by continuous incubation with 5 μg of plasmid with 15 μl of SuperFect (Qiagen) in medium with 10% FCS and antibiotics. The THP-1 cells were treated with 300 nM PMA 18 h before lysing the cells as previously described (29).

Colorimetric assay for β-gal activity

Cells were washed three times with PBS and then lysed with a 0.15% Triton X-100 and 250 mM Tris (pH 8.0) solution followed by freezing and thawing three times. The cell debris was then pelleted, and 25 μl of supernatant was added per well of a 96-well plate (Costar, Cambridge, MA). Enzyme activity was colorimetrically detected by adding 100 μl/well of a solution of 1 mg/ml chlorophenol red galactopyranoside, 60 mM sodium dibasic phosphate (pH 8.0), 10 mM KCl, and 50 μM 2-ME, and absorbance was read at 570 nm. The titration curves for each lysate were compared with a standard curve of β-gal (Boehringer Mannheim, Indianapolis, IN) on each plate using DeltaSOFT II version 3.66 (Biometallics, Princeton, NJ).

ELISA for anti-β-gal IgG

The enzyme-linked immunosorbent assay for Ab to β-gal was previously described (28). The titration curves for each sera were compared with the standard sera arbitrarily set at a maximum of 10^8 U on each plate using DeltaSOFT II version 3.66 (Biometallics).

CTL assay

Splenocytes were harvested and restimulated in vitro with 50 U/ml rIL-2 and peptide for 5 day. Cytolytic T cell assays were performed as described using the CytoTox 96 assay kit (Promega) per the manufacturer's instructions (4). The peptides used included the H-$2K^b$-restricted epitope from OVA, SIINFEKL (30); the H-$2L^d$-restricted epitope from β-gal, TPHPARIGL, (31) and from influenza nucleoprotein the H-$2K^d$-restricted peptide TYQRTRALV; and the H-$2D^b$-restricted peptide, ASNENMETM (32) (Molecular Research Laboratories, Durham, NC). Background controls with an irrelevant peptide for nonspecific target and effector cell lysis were included on every plate. After background subtraction lysis was calculated by: 100 × [(test release-spontaneous release)/(maximum-spontaneous release)].

Results

Membrane-bound Ag can prime CTL responses in DNA immunized mice

To determine whether a nonsecreted Ag was able to induce a CTL response after DNA injection, several plasmid constructs were tested. The first construct encoded the full-length soluble form of OVA, the second encoded OVA fused to the amino terminus of the transferrin receptor (27), and the third plasmid encoded the H-$2K^b$-

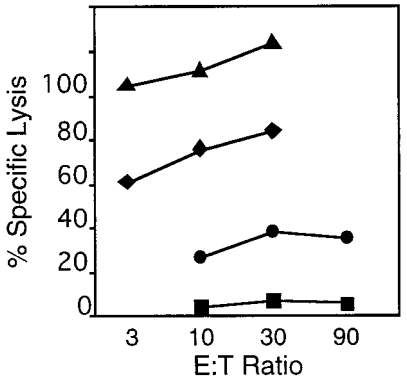

FIGURE 1. Membrane-bound Ag can prime a CTL response after DNA immunization. Four adult female C57BL/6 mice per group were injected with 50 μg of ACB-TPASIINFEKL (●), ACB-OVA (▲), nCMV-TfROVA (♦), and ACB (■) in the rear quadriceps on days 0 and 7. After 2 wk, the mice were sacrificed and their splenocytes assayed for their ability to lyse peptide-pulsed EL4 cells. Values plotted are average peptide-specific lysis. The maximum lysis seen with the negative control peptide (ASNENMETM) was 3.6%. This experiment was performed twice with four mice per group.

restricted epitope from OVA residues 257–264 (SIINFEKL) fused to a leader sequence to facilitate transport into the endoplasmic reticulum (33). The first two plasmids encoded both MHC class I and class II epitopes and the third encoded only a class I-restricted epitope. Mice that were immunized with the membrane-bound form of OVA (TfROVA) were able to elicit a strong CTL response, whereas the minigene-encoding plasmid elicited only a weak response (Fig. 1). The presence of epitopes that could provide cognate T cell help may explain the difference in these two plasmid systems (26, 34). Both plasmid products should have been limited to the cell that was expressing the plasmid, which would imply that APCs were directly transfected. However, in a transgenic model, this membrane-bound form of OVA has been demonstrated to induce autoreactive $CD8^+$ T cells by exogenous Ag transfer (35). Hence, the immunogenic protein may have been expressed by both APCs and other transfected cells.

Plasmid DNA with a macrophage-specific promoter does not induce humoral or cytolytic responses.

To determine whether the cells that were expressing the injected DNA were of monocytic cell lineage, a plasmid with expression restricted to macrophages and related cell types was used (21, 36). A plasmid with *cis*-active elements responsible for transcriptional activation of the scavenger receptor and a β-gal reporter gene was injected i.d. in the tail base of mice (21). This plasmid contained the scavenger receptor proximal promoter and enhancer elements that have been demonstrated to direct macrophage-specific expression in transgenic mice (36). The tissue specificity of this promoter in these transgenic mice limited expression of the reporter gene to spleen, thymus, and testes. No expression was seen in heart, lung, liver, kidney, muscle, or brain. In confirmatory experiments, transfected monocyte lines expressed the β-gal transgene in vitro (Fig. 2*A*). However, mice injected in vivo generated poor anti-β-gal IgG and CTL responses compared with mice injected with a control plasmid with a CMV promoter, pACB-Z, (Fig. 2, *B* and *C*). Similar results were obtained in mice that were injected i.m. with the same plasmids (data not shown).

The weak response from the animals that received the plasmid with the macrophage-specific promoter may have been due to transfection of immature cells with poor Ag-presenting ability. To

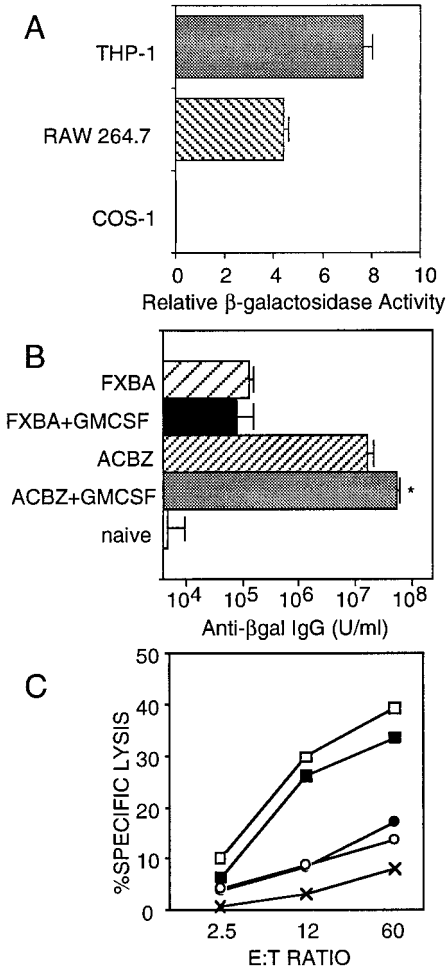

FIGURE 2. Plasmid DNA with a macrophage-specific promoter does not induce humoral or cytolytic responses. Monocyte lines specifically express β-gal after transfection with fXbaSpl-βgal (*A*). In parallel COS-1 (monkey kidney), RAW264.7 (murine monocyte), and THP-1 (human monocytic leukemia) cells were transfected with pACB-Z and fXbaSpl-βgal. The cells were lysed 72 h after transfection and assayed for β-gal activity by colorimetric assay. The data are represented as β-gal activity per cell normalized for transfection efficiency. Control transfections with pACB-Z resulted in β-gal activity of 1328 ng/ml/10^5 in COS cells, 10 ng/ml in RAW and 2.8 ng/ml in THP-1 cells. This experiment was repeated twice. GM-CSF does not augment the weak humoral or CTL response after fXbaSpl-βgal injection. Six adult BALB/c mice per group were injected i.d. in the tail on days 0 and 7 with 50 μg each of fXbaSpl-βgal (●), fXbaSpl-βgal and pCMVGM-CSF (○), pACB-Z (■), pACB-Z and pCMVGM-CSF (□), or nothing (×). Six weeks after immunization, their sera were tested for the presence of anti-β-gal Abs by ELISA (*B*). The IgG response of pCMVGM-CSF- and pACB-Z-coinjected mice was statistically greater than pACB-Z alone ($p < 0.001$ by Student's t test). At 6 wk, the animals were sacrificed and their splenocytes were tested for their ability to lyse peptide-pulsed P815 cells after 5 day of in vitro restimulation (*C*). Values plotted are the mean peptide-specific average background lysis. The maximum lysis seen with the negative control peptide (TYQRTRALV) was 4%. These data are representative of three independent experiments.

address this issue, we attempted to augment the response to this plasmid by coinjecting with a plasmid that expressed GM-CSF. The GM-CSF plasmid coinjection statistically enhanced the Ag-specific IgG response to the pACB-Z control vector but did not improve the response to the plasmid with the macrophage-specific promoter (Fig. 2*B*). Similarly, the CTL response was not improved (Fig. 2*C*). The weak responses indicated that monocyte-derived

cells were not expressing sufficient antigenic protein for the humoral and cellular responses induced after DNA injection.

Directly transfected splenocytes are relatively poor at priming CTL responses

Recent studies using parent into F_1 bone marrow-reconstituted mice demonstrated that the Ag was presented to the immune system in the context of the MHC on the surface of bone marrow-derived cells after plasmid injection (3, 4). It was unclear whether these bone marrow-derived cells were directly transfected or acquired soluble Ag expressed by neighboring transfected somatic cells through macropinocytosis. To separate transfection from protein expression, a tetracycline regulatable system of plasmids was used (22, 23). In this system, the tTA-activated transcription from a minimal promoter sequence from human CMV fused to the *E. coli* tetracycline resistance (*tet*) operator sequences. The tTA was unable to bind to these *tet* operator sequences in the presence of tetracycline, and transcription was suppressed by the drug. The transactivating protein was encoded on a separate plasmid (pUHD15-1) from the regulated transcript (pUHD-OVA). Coadministration of these plasmids allowed efficient transcription of the Ag in the absence of tetracycline.

This plasmid system was used to inject donor mice under drug suppression to minimize the possibility of Ag expression from the injected tissue. The splenocytes from these mice were then transferred to recipients without the drug to allow Ag expression by directly transfected cells that had migrated to the spleens of the donors. Rag-$1^{-/-}$ mice were chosen as donors because there would be no transfer of T or B cells, and their spleens were relatively enriched for APCs (~17% $CD11c^+$ and 50% $MAC3^+$ cells by FACS). The use of TCR transgenic mice, OVA-tcr-1, as recipients increased the sensitivity of detection for the weaker CTL response after injection of tetracycline suppressed splenocytes, which was not seen when wild-type recipients were used (data not shown).

The presence of the injected plasmid was detected by PCR in the proximal part of the tail for all of the donor mice but not the distal tail nor in the spleen (Fig. 3*A*). DNA was successfully isolated from all of the samples as shown in the GAPDH PCR amplification (Fig. 3*B*). The transferred splenocytes from Rag-$1^{-/-}$ mice were relatively enriched for APCs but may not have been a sufficiently purified population to enable amplification of the injected plasmid by PCR (20). The inability to readily detect plasmid in these splenocytes suggested that directly transfected APCs were rare. This paucity of transfected APCs was consistent with reports by investigators who were able to directly visualize such cells by microscopy (7, 8, 19, 37).

One week after the first plasmid injection, the donor Rag-$1^{-/-}$ mice were sacrificed and their splenocytes were transferred as APCs into OVA-tcr-1 mice. Two weeks after transfer, the recipient mice were sacrificed along with naive controls and mice that had been directly inoculated with plasmid DNA. The mice that received splenocytes from donors not on tetracycline mounted a CTL response similar in magnitude to mice that were directly plasmid injected. The splenocytes from these donors could have acquired Ag expressed by dermal keratinocytes or by direct transfection. Splenocytes from tetracycline treated Rag-$1^{-/-}$ mice were not as immunogenic (Fig. 3*C*). These cells could not have acquired transferred Ag in the donor mice but were able to freely express protein from directly transfected cells in the recipient mice. Although the splenocytes from unsuppressed donors had 1 wk longer to express Ag, these data suggest that the predominant mechanism of initiating a CTL response was through cross-priming rather than through directly transfected APCs.

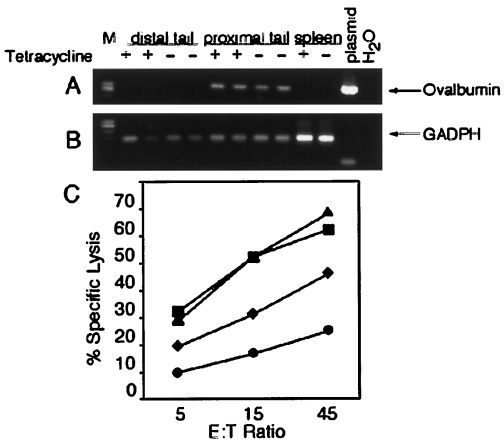

FIGURE 3. Protein expression by nonlymphoid cells is required for CTL priming from i.d. plasmid injection. Two Rag-$1^{-/-}$ mice had 15 mg slow release tetracycline pellets s.c. implanted before injection. These and two other Rag-$1^{-/-}$ mice were injected i.d. in the distal tail with 50 μg each of pUHD-OVA and pUHD15-1 twice, 3 day apart. One week after the first injection, the animals were sacrificed, and 1.3×10^7 splenocytes were transferred i.p. into OVA-tcr-1 mice. DNA was extracted from the pooled spleens and distal and proximal tails of the Rag-$1^{-/-}$ donor mice and then tested for the presence of OVA (*A*) and GAPDH (*B*) genes by PCR. As a positive control, pACBOVA was used, and no DNA was used as a negative control. After 2 wk, the OVA-tcr-1 mice were sacrificed, and their splenocytes were tested for their ability to lyse peptide-pulsed EL4 cells after 5 day of in vitro restimulation (*C*). The average percentage-specific lysis for groups of four OVA-tcr-1 mice are plotted. Represented are mice injected with splenocytes from tetracycline-treated Rag-$1^{-/-}$ mice (♦), with splenocytes from Rag-$1^{-/-}$ mice without drug treatment (■), with pUHD-OVA and pUHD15-1 directly injected (▲), or uninjected mice (●). The maximum lysis seen with the negative control peptide (ASNENMETM) was 3%. This experiment was performed twice.

Intact skin is required for CTL priming from i.d. gene vaccination

To evaluate the role of protein expression by the cells at the site of DNA injection in priming an immune response, mice were injected i.d. in the distal tail with a combination of plasmids encoding soluble Ag and tTA with and without tetracycline suppression. After 1 wk, the tails of some of the mice were proximally amputated and the drug suppression was removed (Fig. 4). If CTL were primed by cells that migrated to a draining lymph node or were transfected at a remote site, then animals that had their tails amputated should have been able to prime an immune response once suppression of gene expression was removed. Control animals that were initially on tetracycline, and had their tails left intact, were able to mount a CTL response once the drug was discontinued. However, mice that had their tails amputated before the removal of drug suppression did not generate a CTL response, demonstrating that protein locally expressed at the site of DNA inoculation was processed for presentation by the immune system.

Immunogenic protein is predominantly expressed by nonlymphoid tissue after DNA immunization

Transgenic mice (TettTALuc) that expressed tTA and luciferase were used to generate bone marrow chimeras with wild-type C57BL/6 mice (24). These mice were lethally irradiated and reconstituted with bone marrow from the other strain. After recovery groups of chimeras and controls were injected i.d. in the tail with pUHD-OVA. After injection with pUHD-OVA, the tissue that concomitantly expressed the tTA transactivator should have been able to express high levels of OVA. The tTA-transgenic mice

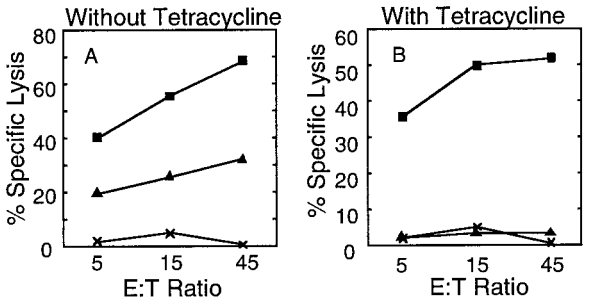

FIGURE 4. Intact skin is required for CTL priming from i.d. gene vaccination. Groups of adult female C57BL/6 mice had 15 mg slow release tetracycline pellets s.c. implanted 5 day before injection. The mice were injected i.d. in the distal tail with 50 μg each of pUHD-OVA and pUHD15-1 twice, 3 day apart. One week after the first injection, the tail was amputated. The tetracycline pellets were then removed. After another week, the mice were sacrificed and their splenocytes were harvested and were then tested for their ability to lyse peptide-pulsed EL4 cells after 5 day of in vitro restimulation. The average percentage-specific lysis for animals without suppression (*A*) and those treated with tetracycline (*B*) are shown. Groups included those with intact tails (■), amputated tails (▲), or untreated mice (×). The maximum lysis seen with the negative control peptide (ASNENMETM) was 16%. This experiment was repeated three times with three to four mice per group.

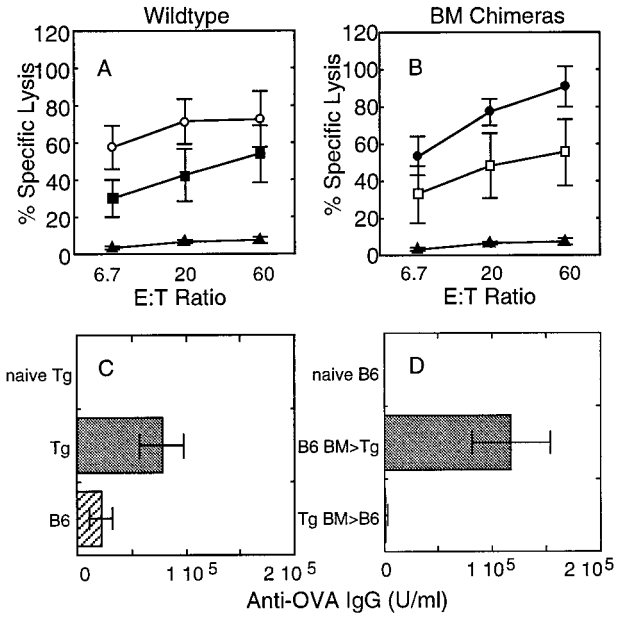

FIGURE 5. Immunogenic protein encoded by injected plasmid DNA is predominantly expressed by nonlymphoid tissue. C57BL/6 and C3-TgN(TettTALuc) mice were lethally irradiated and reconstituted with bone marrow (BM) from the other strain. After reconstitution and recovery, chimeras and controls were injected on days 0 and 7 with 50 μg of pUHD-OVA i.d. in the tail. Six weeks after injection, their splenocytes were removed tested for their ability to lyse peptide-pulsed EL4 cells after 5 day of in vitro restimulation. The average specific lysis ± SEM for TettTALuc (Tg) mice (○), C57BL/6 (B6) mice (■) (*A*), TettTALuc mice with C57BL/6 BM (●) C57BL/6 mice with TettTALuc BM (□) (*B*), and naive mice (▲) are plotted. The maximum lysis seen with the negative control peptide (ASNENMETM) was 7.4%. Four weeks after injection, the mice were bled and their sera analyzed for anti-OVA IgG by ELISA (*C* and *D*). The Ab titers for TettTALuc mice (*C*) and B6 BM into Tg TettTALuc chimeras (*D*) were significant ($p < 0.04$, by Student's *t* test). These data are representative of three experiments.

made substantial anti-OVA CTL and Ab responses regardless of their bone marrow origin (Fig. 5). Conversely, wild-type or chimeric C57BL/6 recipients made weaker CTL and Ab responses. A strong immune response correlated with expression of the transactivator by nonlymphoid tissues and not to bone marrow origin. The locally transfected cells in the tail that contained the transgenic transactivator expressed the protein responsible for generating the immune response.

Discussion

Direct injection of naked plasmid DNA either i.m. or i.d. induces strong, long-lived immune responses to the Ag encoded by the gene vaccine. Both routes of immunization lead to production of Ab and the activation of both MHC class I-restricted, Ag-specific CTL and MHC class II-restricted Th cells secreting Th1-type cytokines (1, 2, 28, 38–40). These properties have made plasmid DNA vaccination an attractive alternative to conventional protein immunization. Consequently, DNA based vaccines are actively being investigated as therapies or preventive measures in such diverse areas as infectious disease, allergy, and cancer (28, 38, 41–43).

In this paper, the cellular mechanisms involved in priming a CTL response after the injection of naked plasmid DNA were further investigated. In a series of experiments, several specialized plasmid systems were used to elucidate the relative roles of resident somatic and migratory bone marrow-derived cells in priming an immune response. The tetracycline responsive promoter system has been demonstrated to tightly regulate gene expression and has enabled investigators to temporally manipulate the expression of transgenes in mice (23, 44–46). This control of expression separated the issues of protein transfer from plasmid transfer. The splenocytes from plasmid injected tetracycline treated Rag-$1^{-/-}$ donor mice were less immunogenic, suggesting that Ag was exogenously acquired rather than exclusively endogenously produced by APCs that migrated to the spleen.

In a follow up study, the site of injection was ablated while plasmid expression was inhibited by tetracycline. If plasmid DNA had transfected cells remote from the site of injection or had been taken up by migratory cells, then there should have been a CTL response after the site of injection was removed and drug suppression was lifted. In control mice that had their tails left intact, sufficient protein was expressed to induce an immune response after tetracycline was discontinued. The inability of mice to raise a CTL response after tail amputation and removal of drug suppression indicated that the cells in the tail skin were responsible for expressing antigenic protein to prime the CTL response.

In a previous report in which muscle and skin injection sites were rapidly ablated after DNA inoculation, the expression of the plasmid was not regulated (47). Immune responses still occurred if injected muscle tissue was excised within 1 min; however, a full response after i.d. immunization required that the injected tail skin remain in place for 72 h before excision. Our results similarly demonstrated that intact skin at the injection site was necessary for mounting an immune response to plasmid DNA encoded Ag. These data argue against CTL being primarily primed by directly transfected cells in the regional lymph nodes after i.d. needle injection of plasmid DNA. The development of an immune response after muscle ablation within 1 min after injection vs a cutaneous immunization suggested that there were likely differences in the extent of transfection outside of the target tissue with technique of DNA administration and site of injection.

Ag expressing and plasmid containing dendritic cells from the skin have been found in draining nodes and in the spleen (7, 8, 19, 37). Although few cells expressing the Ag encoded by the plasmid

were visualized, a large population of untransfected dendritic cells were noted to also migrate to the draining lymph node (7, 8). These untransfected dendritic cells could have acquired Ag expressed by other cell populations in the skin and still prime immune responses. To evaluate whether the immunogen-expressing cells in the skin were exclusively of monocytic cell lineage, a plasmid was used that contained a cell type-specific promoter. The untranslated elements in this plasmid have been previously described to restrict expression primarily to macrophages in the in vitro transfection assays and in transgenic mice (21, 36). In experiments using needle-injected DNA, the cells that expressed immunologically relevant Ag in both muscle and dermally injected mice were not of monocytic cell lineage. The resident connective tissue cell population may be more susceptible to transfection and express protein that is then transferred to migratory bone marrow-derived cells.

As an alternative to endogenous protein expression by APCs, the immune system has the flexibility to acquire extracellular Ag and process it into fragments presented by both MHC class I and class II molecules (12, 13, 15, 16, 35, 48). Such cross priming has been demonstrated for ex vivo plasmid-transfected myoblasts reimplanted i.p. into F_1 mice (18). Also, the skin from gene gun-treated mice stimulated a primary immune response when grafted onto naive recipients (49). There were most likely redundant mechanisms for the intercellular transport and transfer of protein. Different heat shock proteins with roles as chaperones have been shown to induce tumor-specific immunity (50, 51). As part of the reactive response to injury, these heat shock proteins may have become up-regulated after DNA injection and then performed a dual role in transferring Ag to professional APCs.

Alternatively dendritic cells have been demonstrated to engulf apoptotic cells and cross-prime their peptides (52). There may be nonlymphoid cells that expressed protein after DNA inoculation before they underwent apoptosis and were phagocytosed by immature dendritic cells. Transfected keratinocytes have been demonstrated to transfer Ag after irradiation induced cell death (19). The presence of directly transfected hematopoetic cells from DNA injection has been described as short-lived (19). However, Ag in muscle and skin has been found to continue to be expressed relatively long term. The long-lived immune responses may reflect the ability of plasmid DNA to continue to produce low levels of Ag over a prolonged period of time. The nonlymphoid cells were working in concert with professional APCs not merely as innocent bystanders but as Ag generators.

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