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# Analysis of immune responses to varicella zoster viral proteins induced by DNA vaccination

Allison Abendroth [a], Barry Slobedman [b], Matthew L. Springer [c], Helen M. Blau [c], Ann M. Arvin [a,*]

[a] *Department of Pediatric Infectious Diseases, Stanford University School of Medicine, Room G312, Stanford, CA 94305-5208, USA*

[b] *Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305-5208, USA*

[c] *Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305-5208, USA*

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**Abstract**

In this study we sought to examine the mechanism by which immune responses were induced following intramuscular injection of mice with DNA expression vectors encoding genes of varicella zoster virus (VZV). Both VZV-specific antibody and T cell proliferative responses were induced by immunization with DNA sequences for the immediate early 62 (IE62) and glycoprotein E (gE). The viral proteins were shown to be expressed in non-regenerating, rather than regenerating muscle cells. After primary immunization, muscle cells did not express major histocompatibility complex (MHC) class II transcripts and little inflammatory response was detected at the site of inoculation. Histochemical staining and non-isotopic in situ hybridization demonstrated that a second injection of IE62 plasmid DNA was again associated with protein synthesis in non-regenerating muscle cells but that a marked inflammatory infiltrate was induced in muscle tissue. These cells, but not muscle cells, expressed MHC class II transcripts. Significantly, PCR analyses demonstrated that IE62 DNA localized specifically to local draining lymph nodes following primary DNA immunization by intramuscular inoculation. These experiments indicate that transport of plasmid DNA to sites of antigen presentation in regional lymphoid tissue may play an important role in the initial generation of immune responses and that enhancement by secondary inoculation is mediated by immune cells that traffic to the site of viral protein synthesis in muscle cells. 

*Keywords:* Immune responses; Varicella zoster; Viral proteins; DNA vaccination

## 1. Introduction

DNA vaccines have been heralded as a new immunization strategy. Inoculation with plasmid DNA generates protective antibody and cell-mediated immune responses in a variety of animal

* Corresponding author. Tel.: +1-650-7235682; fax: +1-650-7258040.

*E-mail address:* arvinam@leland.stanford.edu (A.M. Arvin)

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models of bacterial, parasitic and viral disease, including herpesviruses (Gregoriadis, 1998). The delivery of plasmid DNA either by direct intramuscular (i.m.) injection into muscle, or intradermally by particle bombardment (gene-gun) induces long lived immune responses. Both routes of immunization have been shown to lead effectively to production of antibodies and the activation of both major histocompatibility complex (MHC) class I-restricted antigen-specific cytotoxic T lymphocytes (CTLs) and MHC class II-restricted $CD4^+$ T cells (Pardoll and Beckerleg, 1995).

The role of muscle cells in the generation of immune responses to plasmid encoded antigens remains poorly understood. The inoculation of *Lac Z* expression vectors has demonstrated that striated muscle cells are the only cell type in which plasmid encoded protein products are detectable (Wolff et al., 1990). Although the precise mechanism of DNA uptake by muscle cells has yet to be elucidated, plasmid DNA has been shown to persist and genes are expressed without replication or incorporation into the host genome for several months after injection (Wolff et al., 1992). The prolonged stability of plasmid vectors is presumably due to the post-mitotic state of muscle cells in vivo (Hohlfeld and Engel, 1994). However, muscle cells have not been previously defined as functioning in vivo as inducers of either primary MHC class I (CTL) or MHC class II (T-helper) responses. Although muscle cells express low levels of MHC class I (Hohlfeld and Engel, 1994) which can be upregulated in vitro by IFN-$\gamma$ (Garlepp et al., 1995), they do not express the essential co-stimulatory molecules B7-1 and B7-2, which are required for the induction of a primary CTL response (Hohlfeld and Engel, 1994; June et al., 1994). In addition, muscle cells in vivo do not express detectable MHC class II molecules. Recent studies have shown that other cell types such as bone marrow derived APCs are likely to play an important role in the generation of immune responses to plasmid encoded proteins following i.m. injection (Xiang and Ertl, 1995; Corr et al., 1996; Doe et al., 1996; Ulmer et al., 1996; Torres et al., 1997).

The purpose of this study was to examine mechanisms by which injection of muscle with plasmids encoding genes of varicella-zoster virus (VZV) induced immunity in a mouse model. Experiments were done using plasmids encoding VZV immediate early 62 (IE62) protein and glycoprotein E (gE).

VZV is a human herpes virus which causes chicken pox (varicella) during primary infection of the host, establishes a state of latency in dorsal root ganglia and may reactivate to cause shingles (herpes zoster) (Arvin, 1995). The IE62 protein is the major virion tegument protein and transactivates viral gene expression (Inchauspe et al., 1989; Arvin, 1995). VZV gE is the most abundant viral glycoprotein. Both IE62 and gE are immunodominant proteins recognized by IgG antibodies and T-lymphocytes from healthy immune subjects (Arvin, 1995). Furthermore, immunization of guinea pigs with IE62 protein has been shown to reduce the occurrence of cell-associated viremia during primary VZV infection as well as the frequency with which VZV reaches dorsal root ganglia (Sabella et al., 1993). Therefore, gE and IE62 are candidates to be considered as VZV vaccine components.

In this study we examined events in the induction of immune responses by direct visualization of the site of injection to define cell types expressing IE62 and gE proteins and to characterize the infiltrating response to primary and secondary injections. In addition, we assessed non-muscle tissues by PCR to determine whether plasmid DNA could traffic to other tissue types after i.m. injection. We also determined whether co-injection of a plasmid expressing IFN-$\gamma$ enhanced VZV specific immune responses and assessed the expression of MHC class II RNA in injected muscle by non-isotopic in situ hybridization.

## 2. Materials and methods

### 2.1. Mice

Female BALB/c mice, aged 4–6 weeks were obtained from the Department of Comparative Medicine, Stanford Medical Center. All animals

were handled in accordance with guidlines of the Administrative Panel on Laboratory Animal Care of Stanford University.

### 2.2. Cells

Vero (African green monkey) cells were grown in DMEM supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Gibco, Gaithersburg, MD), 50 IU of penicillin and 50 μg of streptomycin (Pen/Strep; ICN Biomedicals, Costa Mesa, CA), 0.5 μg of amphotericin B (Fungizone; Flow Laboratories, McLean, VA).

### 2.3. Plasmids

Plasmids pMS62 (Perera et al., 1992a,b) and pCMV5-VZVgE (Litwin et al., 1992) contain the IE62 and gE coding sequences, respectively, under the control of the human cytomegalovirus (HCMV) immediate-early (IE) promoter. These plasmids were shown by transient transfection of Vero cells and immunofluorescence to direct the synthesis of IE62 and gE proteins. pON2345 contains the HCMV IE promoter without any VZV genes and pON249 contains a *Lac Z* cassette under the control of the HCMV IE promoter (kindly provided by Dr E. Mocarski, Stanford University, CA). pBSEA$^k$ was constructed by cloning a 725 bp SmaI-HindIII fragment (containing the H-2EA$^k$ cDNA) from pGem-EA$^k$ (kindly provided by Dr M. Davis, Stanford University, CA) into pBluescript-SK. Transcripts from the T7 promoter are complementary (antisense) to EA$^k$ transcripts. pGIFN-γ was constucted by inserting a 450 bp XhoI–BamHI fragment from pcOVA-γIFN (Maecker et al., 1997), containing the entire murine IFN-γ cDNA, into the EcoRI-BamHI sites of pON2345. The resulting construct contains the murine IFN-γ coding sequence under the control of the HCMV IE promoter; expression of IFN-γ was confirmed by ELISA testing of culture supernatants from transfected Vero cells.

### 2.4. Plasmid DNA injection

Mice were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). The tibialis anterior (TA) muscle was exposed by a single incision in the overlying skin and plasmid DNA was injected into the belly of the muscle using a Hamilton syringe. Both the left and right TA muscles were injected with 50 μg of plasmid DNA in a volume of 10 μl in PBS. The overlying skin was then sutured closed. For immune response experiments, mice were injected on day 0 and day 7 with 50 μg of plasmid DNA into both TA muscles. Three weeks after the final injection, blood was collected by cardiac puncture for immunoblot analysis and spleens were removed to provide T cells for VZV-specific proliferation assays.

### 2.5. Histological, Lac Z and immunofluorescence staining of frozen muscle sections

TA muscles were removed, embedded in mounting medium (OCT compound, Miles Laboratory, Elkhart, IN) and frozen in liquid nitrogen cooled isopentane. Tissue sections (10 and 30 μm) were collected onto glutaraldehyde activated aminopropyltriethoxysilane (APES) coated slides at 100 μm intervals along the entire length of the TA muscle. Sections (30 μm) from muscles injected with pON249 were fixed and stained for β-galactosidase (β-Gal) expression as previously described (Rando and Blau, 1994). Sections (10 μm) were stained with hematoxylin and eosin (H&E) and mounted in Pro-Texx mounting medium (American Scientific Products, McGraw Park, IL) or used in immunofluorescence studies. Sections for immunofluorescence were fixed in acetone at 4°C for 10 min and airdried for 2 h. VZV IE62 and gE proteins were detected by indirect immunofluoresence using rabbit anti-IE62 and anti-gE polyclonal antibodies, respectively (kindly provided by Dr P Kinchington, University of Pittsburgh). Primary antibodies specific for gE and IE62 were used at a dilution of 1:400 and 1:500, respectively. Binding of primary antibodies was detected using a goat anti-rabbit FITC conjugated antibody, diluted 1:100 (Caltag Laboratories, South San Francisco, CA). All antibodies were diluted in PBS containing 10% normal goat

serum and all reactions were done in a humidified atmosphere at 37°C for 30 min, with a 10 min wash in PBS between each step. After the final wash, slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Analysis and photography was performed on a Zeiss Axiophot microscope.

### 2.6. In situ hybridization for MHC Class II ($EA^k$) transcripts

Tissue sections (10 μm) were hybridized at 55°C with a strand-specific digoxigenin (DIG)-labelled riboprobe generated from $pBSEA^k$ using an in situ hybridization protocol described previously (Arthur et al., 1993). Bound probe was detected using anti-DIG antibody coupled to alkaline phosphatase and developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate according to manufacturer's protocol (Boehringer Mannheim, Germany).

### 2.7. Detection of VZV specific antibodies by immunoblot assay

Immunoaffinity purified VZV IE62 and gE proteins were prepared as described previously (Lowry et al., 1992). Purified proteins and control preparations were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 7% gels, followed by electrotransfer to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Individual nitrocellulose strips were blocked with 5% nonfat milk in PBS and incubated for 1 h at room temperature with either sera from individual immunized mice (1:50), positive control rabbit anti-IE62 polyclonal serum (1:800) or polyclonal human anti-VZV serum (1:1000) diluted in blocking solution. Secondary goat anti-mouse and goat anti-human IgG-horseradish peroxidase conjugates were used for enhanced chemiluminscence (ECL) detection of bound antibodies according to manufacturer's protocol (Amersham, Buckinghamshire, England). Bound antibody was visualized by autoradiography and the molecular weight of visible bands were estimated using protein reference standards (Biorad, Richmond, CA).

### 2.8. VZV-specific T cell proliferation assays

Spleens from immunized or non-immunized mice were removed and disrupted between ground glass slides. Cell suspensions were filtered through a sterile nylon mesh to remove large debris and released cells were separated by Ficoll-Hypaque (Pharmacia, Upsalla, Sweden), washed in RPMI and counted. Splenocytes ($3 \times 10^5$ cells/well) were restimulated in vitro with VZV antigen from VZV infected cells or an uninfected cell control antigen in 96 well microtiter plates in RPMI with 10% FCS. Triplicate wells were incubated with VZV antigen or control antigen at ratios of 1:16, 1:64 and 1:256. After 5 days at 37°C, the wells were pulse labelled with $^3$H-thymidine for 18 h and counted using a liquid scintillation counter. Stimulation index (SI) was calculated as the ratio of mean cpm in triplicate antigen stimulated wells to control wells. Each assay included positive control wells stimulated with phytohaemagglutinin. Mean SI values were calculated and compared using Student's *t*-test: the software package Statview II (Abacus Concepts, CA).

### 2.9. PCR and DNA blot hybridization

Spleens, draining lymph nodes and muscles from immunized or non-immunized mice were removed and disrupted between ground glass slides. Cell suspensions were filtered through sterile nylon mesh to remove large debris and the released cells washed three times in PBS, counted and resuspended in lysis buffer (50 mM KCl, 10 mM Tris–HCl pH 8.5, 2 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween-20, 100 μg/ml proteinase K) at a concentration of $5.2 \times 10^4$ cells per 10 μl. After incubation for 16 h at 65°C and 10 min at 98°C to inactivate proteinase K, 10 μl aliquots of cell lysate were subjected to PCR amplification for 35 cycles (94°C for 1 min, 57°C for 1 min and 72°C for 2 min) with primers IE62-A (GCAGGCCTTCTCCCCTGTAT) and IE62-B (GGGAGTGGGACCTTAACCTT). After amplification, 20% of each reaction product

was resolved by electrophoresis in 3% agarose gels and analysed by DNA blot hybridization as previously described (Slobedman and Simmons, 1997) using a random primed $^{32}$P-labelled probe generated from linearized pMS62 plasmid.

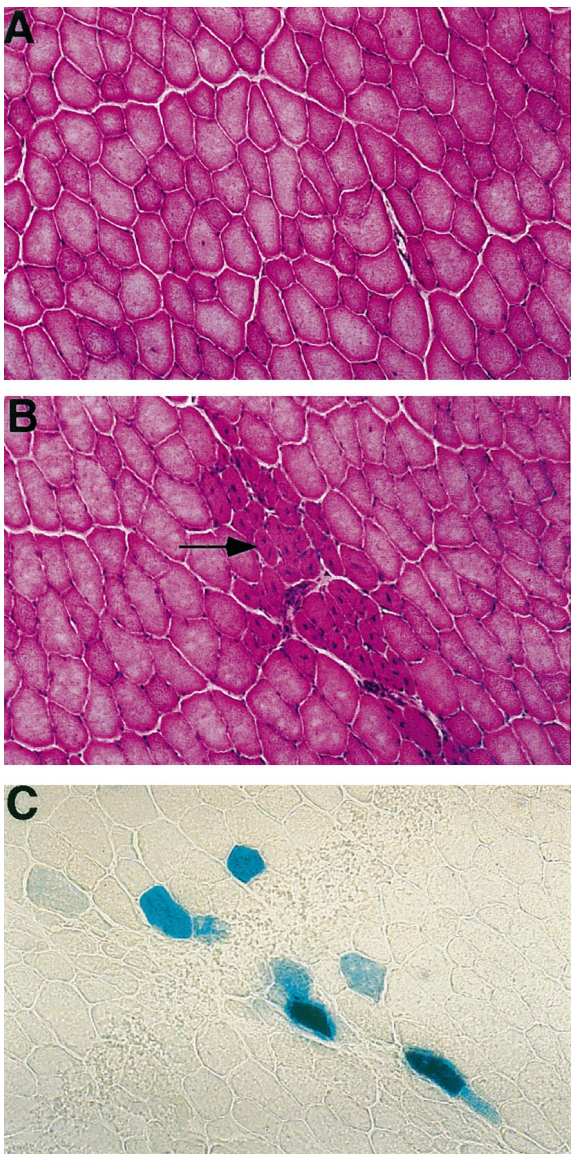


Fig. 1. Histology and β-galactosidase expression in mouse muscle injected with a *Lac Z* expression vector. H&E stained section of an uninjected mouse TA muscle, showing large muscle cells with multiple nuclei distributed around the periphery (A). The injection site is readily distinguished by smaller, centrally nucleated regenerating muscle cells in a localized area which are visualized in a mouse TA muscle 7 days after injection with pON249 DNA (B: arrow). Histochemical detection of β-galactosidase (blue staining) is evident in mouse TA muscles 7 days after injection with pON249 DNA (C).

## 3. Results

### *3.1. Detection of expression of Lac Z, IE62 and gE proteins in mouse muscle cells following DNA injection*

Initial studies involved the direct i.m. injection of mouse TA muscles with 50 μg of pON249 DNA (*Lac Z* expression vector). Seven days after injection, TA muscles were removed, frozen and cryostat sections (10 and 30 μm) were collected for analysis. Ten micrometre sections were H&E stained to examine tissue histology (Fig. 1A and B) and 30 μm sections were stained for β-galactosidase (β-gal) expression (Fig. 1C). Muscle tissue from uninjected animals showed typical morphology with large, tightly packed cells that had multiple nuclei at the margins of the cell membrane. In contrast, in injected muscle tissue, the needle track could be readily distinguished by smaller, centrally nucleated muscle cells in a localized area. These cells had morphologic changes (i.e. centrally located nuclei) which is characteristic of regenerating muscle cells. Staining with 5-bromo-3-chloro-indoyll β-D-galactoside demonstrated the expression of β-galactosidase in cells around the injection site, but not within these regenerating muscle cells. This staining pattern confirmed that pON249 was taken up by and expressed in muscle cells surrounding the injection site.

Indirect immunofluorescence was used to detect and localize IE62 and gE protein expression in mouse TA muscles 7 days after i.m. injection with 50 μg of either pMS62 or pCMV5-VZVgE. Sections (10 μm) of frozen TA muscles were tested for IE62 and gE protein expression using rabbit polyclonal anti-IE62 or anti-gE serum. Negative controls included incubation with an irrelevant primary antibody and muscle sections from mice injected with pON2345. IE62 protein was detected exclusively in the nuclei of intact, non-regenerat-

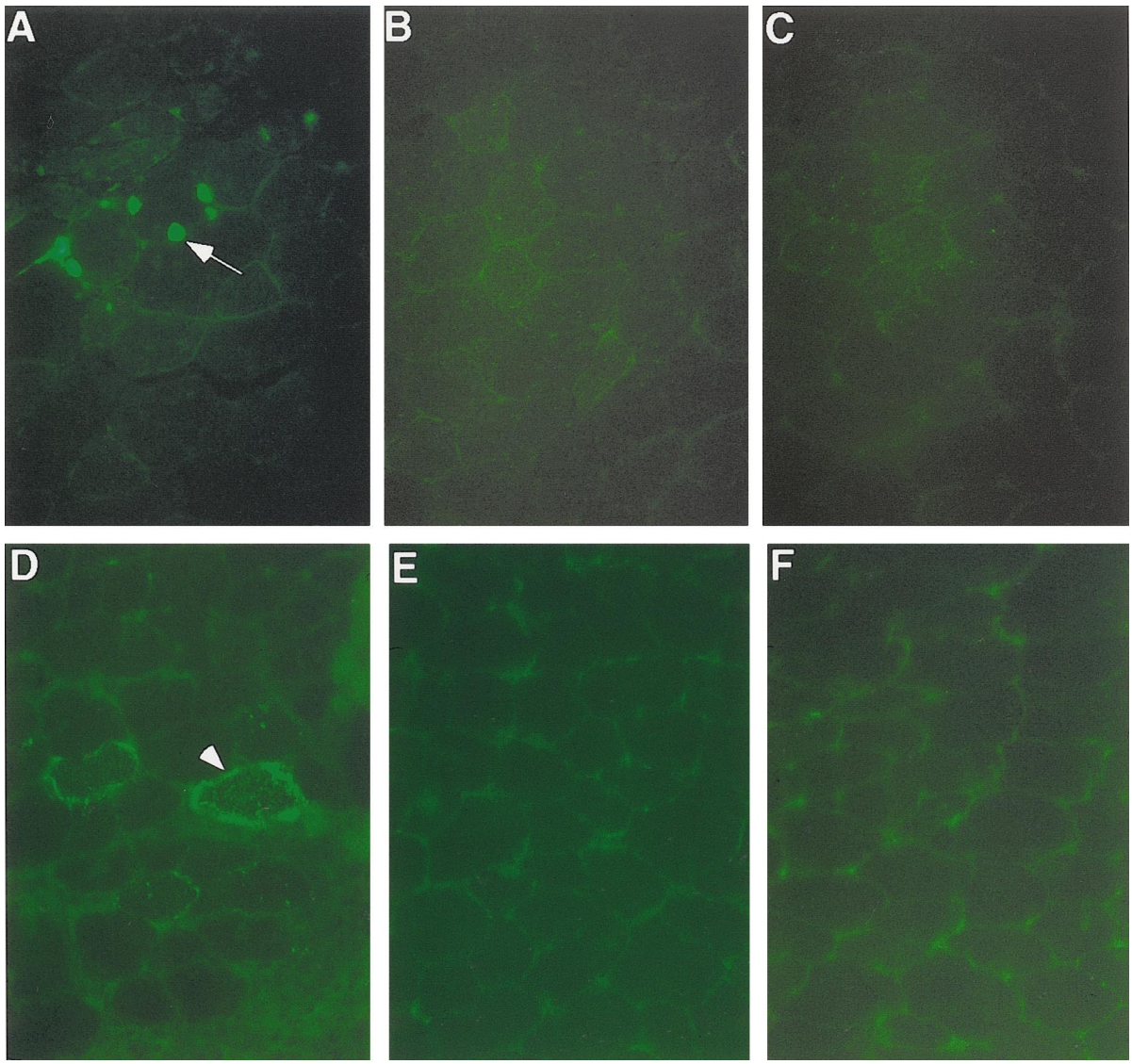


Fig. 2. Expression of VZV IE62 and gE proteins in mouse muscles injected with IE62 and gE expression vectors. Indirect immunofluorescence in sections of mouse TA muscles 7 days after injection with pMS62 (A) and pCMV5-VZVgE DNA (D). Intense nuclear IE62 staining (A: arrow) and cytoplasmic and surface gE staining (D: arrowhead) was detected in intact, undamaged muscle cells adjacent to the injection site. No staining was detected in TA muscle sections from mice injected with pON2345 (C and F) or in sections from mice injected with pMS62 and pCMV5-VZVgE incubated with irrelevant primary antibodies (B and E).

ing muscle cells adjacent to the injection site (Fig. 2A). The nuclear localization of IE62 staining and non-regenerating nature of positive stained cells was confirmed by light and phase contrast microscopy. The prominent staining of cell nuclei was expected because the IE62 sequence encodes a nuclear localization signal. Cytoplasmic and surface staining for gE was detected in non-regenerating muscle cells from pCMV5-VZVgE immunized mice (Fig. 2D). Staining was not detected in sections from mice injected with either pMS62 or pCMV5-VZVgE after incubation with

an irrelevant primary antibody (Fig. 2B and E), or in sections from mice injected with pON2345 (Fig. 2C and F). These data indicate that IE62 and gE proteins were expressed in mouse muscle cells following i.m. injection of plasmids encoding these genes and that expression was localized to non-regenerating cells.

### 3.2. Cellular and humoral immune responses following i.m. injection of IE62 and gE expressing plasmids

To examine cellular and humoral responses to plasmid encoded IE62 or gE, mice were injected in both TA muscles with 50 μg of either pMS62 (ten mice) or pCMV5-VZVgE (five mice). Control mice were injected with pON2345 (15 mice). A second injection of plasmid DNA was delivered 7 days later. We also sought to determine whether co-injection of pMS62 with a plasmid expressing IFN-γ (pGIFN-γ) had any effect on immune responses to IE62. A group of seven mice was injected with both pMS62 and pGIFN-γ (50 μg of each) and a control group of seven mice was injected with both pON2345 and pGIFN-γ (50 μg of each) as described above.

Twenty one days after the final injection, mice were bled and sera screened for IE62 and gE specific antibodies by immunoblot analysis. In addition, splenocytes were tested for a VZV-specific T cell proliferative response.

Splenocytes from nine of ten mice injected with pMS62 and six of seven mice given pMS62/pGIFN-γ showed a VZV-specific T cell proliferative response (Fig. 3A). The mean peak SI for mice injected with pMS62 was $11.2 \pm 2.5$ SEM and $10.9 \pm 2.8$ SEM for mice injected with pMS62/pGIFN-γ. This difference was not statistically significant ($P = 0.936$, Students $t$-test). Splenocytes from mice injected with pON2345 (ten animals) and pON2345/pGIFN-γ (seven animals) had an $SI < 2.2$. In pCMV5-VZVgE injected mice, five of five animals showed a VZV-specific T cell proliferative response (Fig. 3B). These data show that VZV specific T cell proliferative responses were induced following i.m. injection with pMS62 or pCMV5-VZVgE. Co-injection of pGIFN-γ with pMS62 did not enhance VZV specific T cell proliferative responses.

Although T cell responses were predominant, sera from some mice had detectable antibodies to IE62 or gE, when tested using nitrocellulose strips containing affinity purified IE62 or gE and uninfected cell control protein (Fig. 4). Two of ten mice injected with pMS62 and one of seven mice injected with pMS62/pGIFN-γ showed a detectable antibody response to IE62. IE62 antibody was not detected in sera from mice injected with control pON2345 (ten animals) or pON2345/pGIFN-γ (seven animals). In pCMV5-VZVgE injected mice, one out of five animals showed a detectable antibody response to gE. Antibody to gE was not detected in sera from five mice injected with pON2345. Whilst co-injection of pGIFN-γ and pMS62 DNA did appear to influence VZV specific antibody responses, a statistical interpretation was not possible due to the small number of responding animals.

### 3.3. Detection of MHC class II RNA in infiltrating cells following i.m. DNA injection

To determine whether i.m. injection of plasmid DNA influenced the expression of MHC class II in muscle tissue, we performed non-isotopic in situ hybridization for MHC class II transcripts. Mice were injected with 50 μg of either pMS62 or pON2345 on day 0 and day 7 in both TA muscles. Seven days after the second injection, muscles were removed, 10 μm frozen sections were collected onto APES coated slides and either stained with H&E or hybridized with a strand specific DIG-labelled riboprobe designed to detect MHC class II ($EA^k$) transcripts (Fig. 5). H&E staining of pMS62 injected muscle tissue revealed the presence of an extensive inflammatory infiltrate, which was not observed in pON2345 injected muscle or in muscle tissue stained 7 days after a single injection of pMS62. By in situ hybridization, MHC class II transcripts were readily detected in the inflammatory cells infiltrating the pMS62 injected muscle but were not detected in muscle cells. The MHC class II probe did not hybridize to muscle sections from pON2345 injected mice. In addition, a riboprobe generated in the opposite

orientation did not hybridize to infiltrating cells in pMS62 injected muscle, confirming the RNA specificity of the staining. It was concluded that a second injection of pMS62 induced an inflamma-

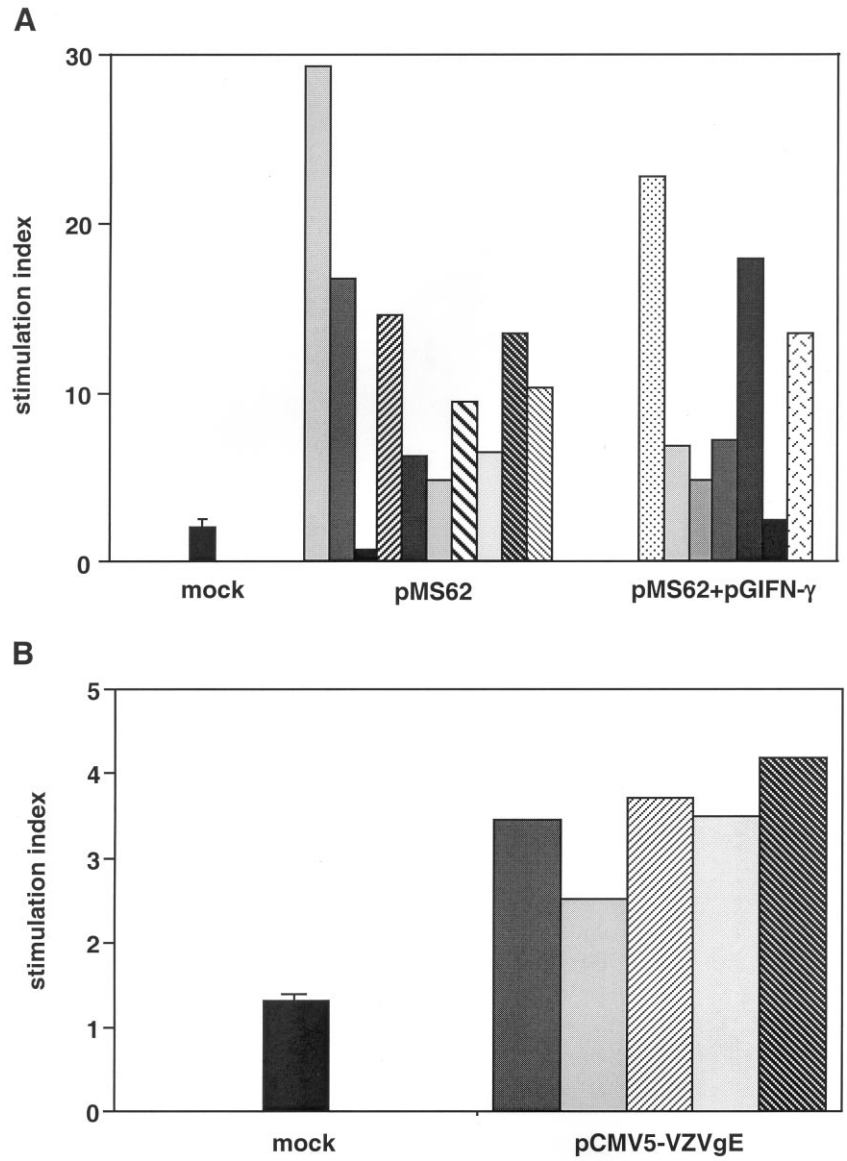


Fig. 3. VZV-specific T cell proliferative responses after DNA injection of mice with plasmids encoding IE62 protein or gE. Three weeks after the final injection, splenocytes from individual mice were stimulated in vitro with VZV antigen or an uninfected cell control. The stimulation index (SI) was calculated as the ratio of mean cpm in triplicate antigen stimulated wells to control antigen wells. Responses of mice injected with pMS62 or pMS62/pGIFN-γ DNA are shown in panel A. The SI of the negative control (mock) group represents the mean peak SI ( + SE) of mice injected with pON2345 or pON2345/pGIFN-γ. SI of mice injected with pCMV5-VZVgE are shown in panel B. The negative control group (mock) represents the mean peak SI ( + SE) of mice injected with pON2345.

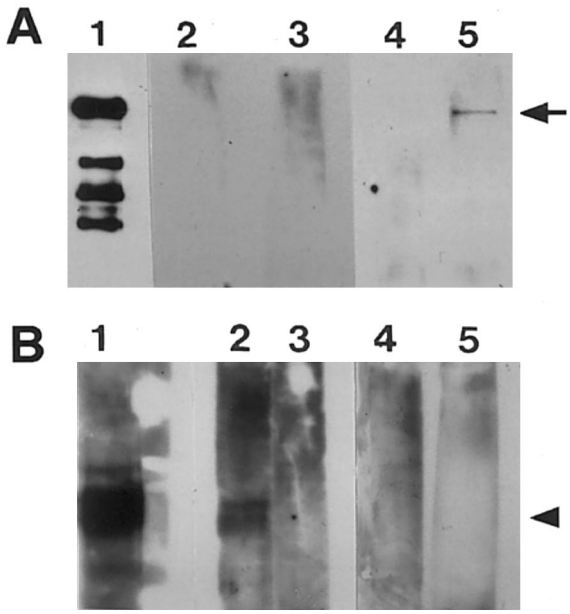


Fig. 4. Detection of serum IE62 and gE antibodies. Individual nitrocellulose strips containing purified IE62 (A: Lanes 1, 3 and 5) or control uninfected cell proteins (A: Lanes 2 and 4) or purified gE (B: Lanes 1, 2 and 4) and control uninfected cell proteins (B: Lanes 3 and 5) were incubated with sera from individual mice (1:50); bound antibodies were detected by ECL. Panel (A) shows a representative immunoblot using sera from mice injected with pMS62 (Lanes 4 and 5) or pON2345 DNA (Lanes 2 and 3). Lane 1 (positive control) illustrates binding of a rabbit anti-IE62 polyclonal serum to purified IE62 protein. The arrow indicates antibody binding to the IE62 protein band in immunized mouse serum (Lane 5). Panel (B) is a representative immunoblot using sera from mice injected with pCMV5-VZVgE (Lanes 2 and 3) or pON2345 DNA (Lanes 4 and 5). Lane 1 illustrates binding of a human anti-VZV polyclonal serum to purified gE protein. The arrowhead indicates antibody to the gE protein bands with immunized mouse serum (Lane 2).

tory infiltrate in muscle tissue and invading infiltrating cells, but not muscle cells, expressed MHC class II transcripts.

### *3.4. Detection of pMS62 DNA in draining lymph nodes following DNA injection*

Failure to detect MHC class II in muscle cells expressing VZV proteins indicated that these cells could not function as direct APCs. To determine whether other tissues besides muscle harbored plasmid DNA following i.m. injection, mice were injected with 50 μg of either pMS62 or pON2345. Seven days after injection, TA muscles, draining lymph nodes and spleens were removed, single cell suspensions prepared, washed thoroughly and incubated in lysis buffer. Cell lysates of $5 \times 10^4$ cells were subjected to 35 rounds of PCR amplification with primers IE62-A and IE62-B, which were designed to amplify sequences from pMS62. $5 \times 10^4$ cells from spleen, draining lymph nodes and muscle from pON2345 injected mice were included as negative controls. A PCR control containing no DNA was also included. After amplification, 20% of each reaction product was separated on a 3% agarose gel, Southern blotted and probed with a random primed $^{32}$P-labelled probe specific for amplified sequences. A 296 bp product was readily visualized in cell lysates from pMS62 injected muscle and draining lymph nodes, corresponding to the expected size of the PCR product derived from pMS62 (Fig. 6). In contrast, no pMS62 products were detected in spleen samples from mice injected with pMS62 or in the muscle, draining lymph node and spleen samples from mice injected with pON2345. These data suggest that, in addition to muscle, pMS62 DNA localized specifically to the draining lymph nodes following i.m. injection of plasmid DNA and persisted for at least 7 days.

## 4. Discussion

These experiments provide the first demonstration of immune responses generated to VZV proteins after i.m. injection of plasmid vectors. Despite the ability to clearly visualize regenerating cells corresponding to the needle track in injected muscle, histochemical and immunofluorescent staining demonstrated that plasmid encoded protein expression occurred exclusively in non-regenerating muscle cells adjacent to the injection site. Davis et al. (1993) were also unable to detect β-gal positive cells in regenerating muscle cells following i.m. injection of a *Lac Z* expression plasmid. IE62 protein has a strong nuclear localization signal and was detected in nuclei whereas gE, which is a glycoprotein, localized to the cytoplasm and surface of muscle cells. Since both IE62 and gE plasmids elicited VZV-specific T cells and

IgG antibodies, the induction of immune responses was independent of the pattern of antigen localization within muscle cells. In this respect, others have also shown that i.m. injection with DNA encoding cytoplasmic or secreted proteins both lead to the induction of effective immune responses (Ulmer et al., 1993; Ertl et al., 1994). The inability to detect antibody responses to either IE62 or gE in some of the animals tested may be a reflection of the sensitivity of the immunblot assay used in these experiments, or alternatively may reflect a lack of induction of an antibody response in these animals.

Although viral protein expression by muscle cells was proved our experiments also show that muscle cells expressing viral proteins exhibited no upregulation of MHC class II expression and the local inflammatory response was minimal after primary DNA injection. These observations suggest that the events required to sensitize T cells to these VZV proteins were not occurring at this site. However, the IE62 plasmid DNA was detected in draining lymph node tissue, and persisted for at least 7 days after a single i.m. injection. These findings support the hypothesis that initial antigen presentation following DNA immunization does not involve antigen synthesis by muscle cells. Instead, plasmid DNA may reach draining lymph nodes by transport as free DNA through the lymphatic system or via motile cells which take up

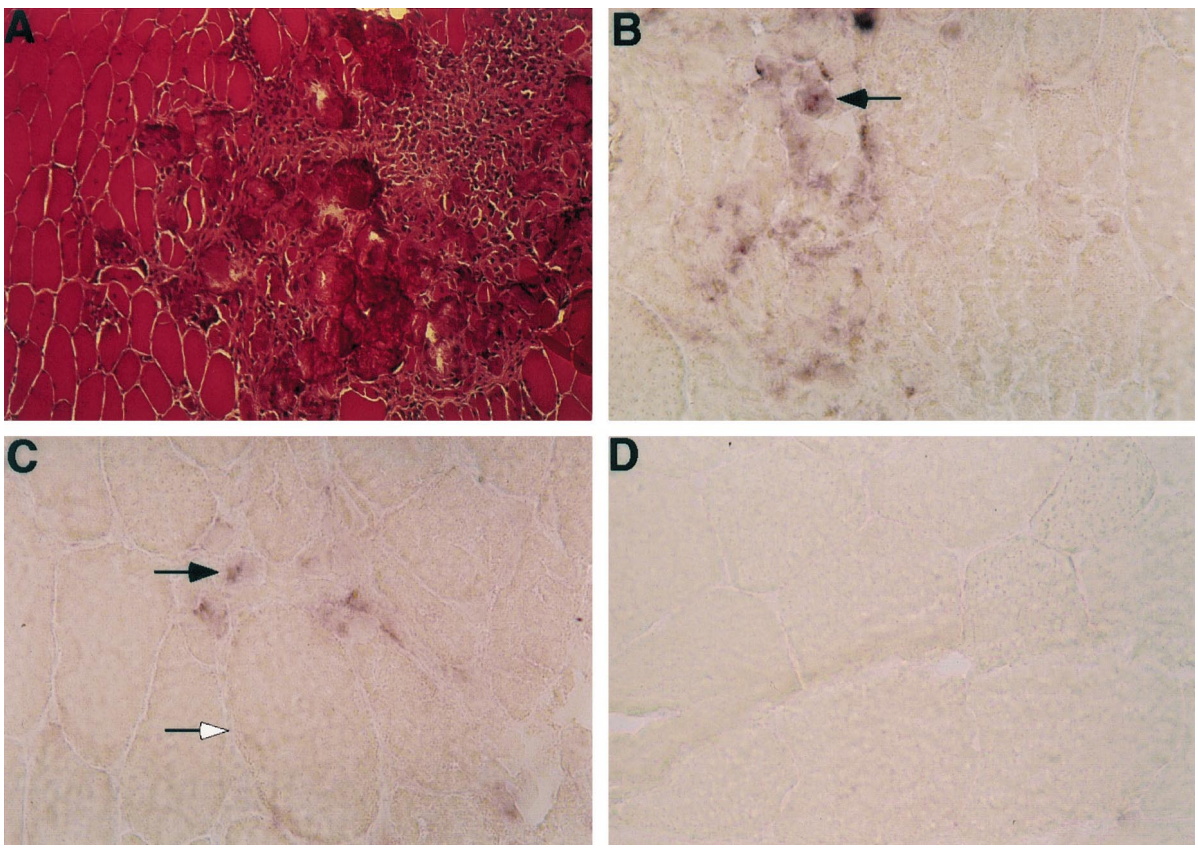


Fig. 5. Infiltrating response and MHC class II RNA expression after sequential injections of the IE62 expression vector. H&E stained section of a mouse TA muscle, 7 days after two injections with pMS62 DNA (injected day 0 and day 7), show inflammatory infiltrating cells (A). TA muscle sections from pMS62 injected mice were hybridized with a strand specific DIG-labelled riboprobe to MHC class II transcripts (B and C). Positive hybridization was detected in the infiltrating cells (black arrow), but not muscle cells (white arrow). No specific hybridization was detected in TA muscle sections from mice injected with pMS62 incubated with an opposite orientation DIG-labelled riboprobe (D).

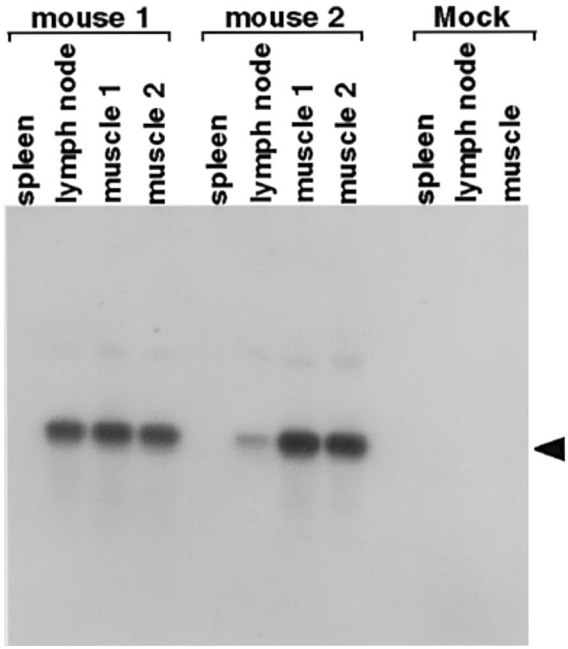


Fig. 6. Detection of IE62 specific DNA sequences by PCR amplification and Southern blot hybridization in muscles and draining lymph nodes after i.m. injection with pMS62. Arrowhead indicates the position of a 296 bp IE62 specific fragment detected in total DNA extracted from muscles and draining lymph nodes from two mice 7 days after injection with pMS62. IE62 specific sequences were not detected in spleens of mice injected with pMS62 or in the spleens, draining lymph nodes and muscles from mice injected with pON2345 (mock).

DNA directly from the site of i.m. injection. In this respect, gene expression from a plasmid vector has been detected in small numbers of dendritic cells in draining lymph nodes after skin scarification of mouse ears (Akbari et al., 1999). Our experiments demonstrated that plasmid DNA trafficked specifically to regional lymph node tissue following i.m. injection. In earlier studies, DNA was not detected in lymph nodes or other organs (Nichols et al., 1995) or detection has been limited to blood plasma at early times, i.e. 4 h, after i.m. injection (Winegar et al., 1996). Our ability to reproducibly detect IE62 plasmid DNA in draining lymph nodes may reflect the enhanced sensitivity of our PCR assay, which has a detection limit of three DNA copies per $5 \times 10^4$ sample cells (data not shown).

Our observations are consistent with the mounting evidence that non-muscle cells play a crucial role in the generation of immune responses after i.m. administration of plasmid DNA. Boyle et al. (1998) demonstrated enhanced humoral and cellular immune responses to plasmid DNAs that encoded antigens directed specifically to lymph nodes and APCs. Torres et al. (1997) showed that surgical excision of mouse TA muscle, removed within 1–10 min after injection, did not affect the magnitude of antigen-specific immune responses to membrane bound, secreted or intracellular expressed antigens. In addition, experiments using a plasmid expressing the rabies virus glycoprotein co-injected with a plasmid encoding GM-CSF, a cytokine known to enhance antigen presentation by dendritic cells, was shown to enhance B and T helper cell responses (Xiang and Ertl, 1995). Experiments by Rodriguez et al. (1997) using ubiquitination of a viral protein indicate that i.m. injection cannot elicit primary immunity as a result of soluble protein release and uptake by APCs. Furthermore, studies using chimeric mice demonstrated that CTL responses induced following i.m. DNA injection were restricted to professional APCs, derived from donor bone marrow, and not the recipient muscle cells (Corr et al., 1996; Doe et al., 1996).

Although muscle cells express low levels of MHC class I in vivo and no detectable MHC class II, it has been reported that IFN-$\gamma$ treatment of muscle cells in vitro results in the upregulation of MHC class I and induction of MHC class II expression (Hohlfeld and Engel, 1994; Garlepp et al., 1995). We were therefore interested in determining whether muscle cells in vivo had the potential to act as APCs by assessing their expression of MHC class II after DNA immunization. Muscle cells showed no detectable induction of MHC class II RNA when injected with DNA encoding IE62 alone, or when the IE62 plasmid was co-injected with an IFN-$\gamma$ expressing plasmid. However, an extensive inflammatory infiltrate was detected in muscle tissue 7 days after a second injection with either IE62 alone, or IE62 and IFN-$\gamma$ expressing plasmids. These infiltrating cells were MHC class II positive, but muscle cells remained negative. The infiltrating cells that express MHC class II are likely to play an important role in the amplification of the

antigen-specific immune response. By this model, T cells that have been primed initially by antigen presentation occurring in regional lymph nodes after the first DNA injection should traffic in large numbers to sites of antigen synthesis in muscle cells, when the second dose of DNA vaccine is given, as we observed in mice injected with the IE62 plasmid. At this point, the recruitment of inflammatory cells that have the capacity to take up locally produced viral proteins, and process the antigen for MHC presentation, should enhance host responses to the foreign protein. Our observations explain the enhanced immunogenecity that has been described with two dose regimes of DNA immunization (Gregoriadis, 1998).

Co-injection of mice with plasmids expressing IE62 and IFN-γ did not result in a significant change in T cell proliferative responses and antibody responses also did not appear to be altered. Our result differed slightly from those reported by Xiang and Ertl (Xiang and Ertl, 1995), who showed that co-injection of a plasmid expressing the glycoprotein of rabies virus with a plasmid expressing IFN-γ resulted in a small decrease in the immune responses to the viral antigen when antibody responses were measured by ELISA and T cell sensitization was assessed by lymphokine release assays. They postulated that this decrease may have resulted from IFN-γ induced upregulation of MHC class I molecules, which may have improved the recognition by CTLs, resulting in the lysis of antigen-expressing cells followed by termination of the immune response. In our study, histological examination of muscle tissue 7 days after a single injection did not reveal any detectable inflammatory infiltrate or muscle cell damage apart from the needle track itself. Whilst we detected a large infiltrate of MHC class II positive cells in muscle tissue expressing viral proteins after a second DNA injection, we did not observe extensive muscle cell damage and protein expression was evident only in non-regenerating muscle cells. However, it is possible that immune-mediated lysis of a few protein-expressing cells did occur. If so, released antigens may have been taken up directly by dendritic cells or macrophages as suggested by Davis et al. (1997). The studies of Ulmer et al. (1996) indicated that transfer of antigen from muscle cells to professional APCs is likely to be a factor in DNA vaccine immunogenicity.

Other investigators have demonstrated that i.m. DNA injection is an effective method to induce immunity against proteins derived from other herpes viruses, with a prominent T cell response, as we observed to VZV IE62 and gE (Cox et al., 1993; Manickan et al., 1995a,b; Pande et al., 1995; Bourne et al., 1996a,b; Gonzalez Armas et al., 1996; Kriesel et al., 1996; Monteil et al., 1996; McClements et al., 1996; Kuklin et al., 1997). Several groups have also demonstrated that DNA encoding herpes simplex virus proteins can induce anti-viral protection in animal models of infection (Manickan et al., 1995a,b; Kriesel et al., 1996; Bourne et al., 1996a). Whilst we might expect that the immune responses generated to VZV proteins would provide at least some degree of protection to VZV challenge, we did not assess anti-viral protection because VZV is highly species specific and is not infectious for mice. Immunity elicited by IE62 and gE is protective against challenge in guinea pigs (Lowry et al., 1992; Sabella et al., 1993).

In summary, VZV proteins are capable of inducing humoral and cellular immune responses after i.m. injection of plasmid DNA. Our experiments link prior observations about the mechanisms by which DNA vaccines elicit immunity by demonstrating the persistence of the foreign gene in draining lymph nodes, allowing for initial antigen presentation at this site, and the prolonged expression of viral protein by muscle cells, which should facilitate the clonal expansion of antigen-specific T cells and the generation of a sustained host response to the foreign protein.

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