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Intradermal gene immunization: The possible role of DNA uptake in the induction of cellular immunity to viruses

(influenza/transfection/antigen-presenting cells/genetic vaccination)

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ABSTRACT **The skin and mucous membranes are the anatomical sites where most viruses are first encountered by the immune system. Previous experiments have suggested that striated muscle cells are unique among mammalian cell types in their capacity to take up and express free DNA in the absence of a viral vector or physical carrier. However, we have found that mice injected into the superficial skin with free (naked) plasmid DNA encoding the influenza nucleoprotein gene had discrete foci of epidermal and dermal cells, including cells with dendritic morphology, that contained immunoreactive nucleoprotein antigen. A single intradermal administration of 0.3–15 μg of free plasmid DNA induced anti-nucleoprotein-specific antibody and cytotoxic T lymphocytes that persisted for at least 68–70 weeks after vaccination. Intradermal gene administration induced higher antibody titers than did direct gene injection into skeletal muscle and did not cause local inflammation or necrosis. Compared with control animals, the gene-injected mice were resistant to challenge with a heterologous strain of influenza virus. These results indicate that the cells of the skin can take up and express free foreign DNA and induce cellular and humoral immune responses against the encoded protein. We suggest that DNA uptake by the skin-associated lymphoid tissues may play a role in the induction of cytotoxic T cells against viruses and other intracellular pathogens.**

The injection of free (naked) plasmid DNA (pDNA) into skeletal muscle can lead to protein expression and to the induction of cytotoxic T cells (CTL) and antibodies against the encoded protein antigens (1–4). Striated muscle is considered to be the only tissue that efficiently takes up and expresses pDNA in the absence of a viral vector or a physical carrier (5). However, muscle is not considered to be a site for antigen presentation because it contains few if any dendritic cells, macrophages, and lymphocytes. The skin and mucous membranes are the anatomical sites where most exogenous antigens are normally encountered. The skin-associated lymphoid tissues contain specialized cells that enhance immune responses. The keratinocytes produce interleukin 1 and tumor necrosis factor α, which can activate lymphocytes, macrophages, and dendritic cells (6). The Langerhans' cells of skin carry the antigen from the skin to the draining lymph nodes. Antigen-loaded Langerhans' cells are potent activators of naive T lymphocytes (7). A special subset of circulating T lymphocytes (epidermotropic lymphocytes) homes to the skin and plays a critical role in cutaneous immunity (8). The dendritic cells and macrophages of the dermis can also take up antigen and initiate immune responses. Therefore, the *in vivo* transfection of epidermal or dermal cells by DNA would be expected to provide an efficient route for gene immunization that mimics a physiologic response to infection.

In an attempt to overcome the apparent inability of skin cells to take up and express pDNA, gold microprojectiles have been used to physically deliver genes into the cytoplasm of skin cells (9, 10). Recently, we fortuitously observed that the injection of pDNA into the skin could trigger a humoral immune response (11). Because the needle passed through the skin, with inevitable intradermal (i.d.) leakage of some DNA, we reconsidered the possibility that skin cells might be capable of taking up free DNA, expressing the encoded protein, and initiating an immune response. The experiments reported here verify this hypothesis and raise the possibility that DNA uptake and processing by the skin-associated-lymphoid tissue may be an adaptive mechanism for the efficient induction of cellular immunity to viruses.

METHODS

Plasmid. The expression vector pRSV-NP contains the Rous sarcoma virus (RSV) long terminal repeat as a promoter element, the nucleoprotein (NP) gene from influenza strain A/PR/8/34 (H1N1), the simian virus 40 virus t-intron, and the simian virus 40 polyadenylylation site. The pCMV-NP plasmid is the same as pRSV-NP, except that the RSV promoter was replaced by the cytomegalovirus (CMV) IE1 promoter-intron (1, 3). The pCMV-BL control plasmid is the basic expression vector without the NP gene. pDNA was purified by centrifugation through CsCl and was stored frozen in 10 mM Tris·HCl/0.1 mM EDTA, pH 8.0. The endotoxin content was 5 ng/mg of DNA as determined by limulus amebocyte lysate assay (Sigma). Before injection, the plasmid was precipitated in ethanol, washed with 70% (vol/vol) ethanol, and dissolved in normal saline.

Gene Injection. With a 1-ml syringe and a 28-gauge needle, BALB/c mice were injected either intradermally (i.d.) or intramuscularly (i.m.). i.d. inoculations were 1–2 cm distal from the tail base, with various amounts of free pDNA in a total volume of 100 μl, and i.m. DNA injections were as described (1, 3, 4).

Antibody Assays. IgG anti-NP antibodies were measured by ELISA, using recombinant NP as the solid-phase antigen, according to the method described by Rhodes *et al.* (1). Briefly, microtiter plates (Costar) were coated with 2.5 μg of antigen per ml of borate buffer saline (BBS; 90 mM borate, pH 8.3/89 mM NaCl) overnight at 4°C, blocked for 1 hr with bovine serum albumin at 10 mg/ml. Serum samples were serially diluted in BBS starting at 1:40, added to the plate, and

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Abbreviations: NP, nucleoprotein; CTL, cytotoxic T cells; pDNA, plasmid DNA; CMV, cytomegalovirus; RSV, Rous sarcoma virus; i.d., intradermal or intradermally.

stored overnight at 4°C. Plates were washed with BBS/0.05% Tween 20 and then incubated with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch) for 2 hr at room temperature. Plates were washed again, and a solution of *p*-nitrophenol phosphate (1 mg/ml) (Boehringer Mannheim) in 0.05 M carbonate buffer, pH 9.8, 1 mM $MgCl_2$ was added. Absorbance at 405 nm was read 2 hr after substrate addition.

Cytotoxicity Assay. Splenocytes from immunized animals (responders) were isolated 70 weeks after injection and cultured with irradiated syngeneic spleen cells (stimulators) that had been incubated with NP peptide (10 μg/ml, amino acid sequence TYQRTRALV), representing the major K^d epitope (12), for 30 min at 37°C and added to effectors cells after three washes. Approximately 2.5×10^7 responders and stimulators were placed together in flasks in 25 ml of RPMI 1640 medium/10% fetal bovine serum/50 μM 2-mercaptoethanol. Twenty-four hours later, human recombinant interleukin 2 (Boehringer Mannheim) at 1 unit/ml was added. After 6 days of culture, standard 6-hr ^{51}Cr-release assays were done in microtiter plates. The targets were ^{51}Cr-labeled P815 (H-2^d) mastocytoma cells, at a density of 1×10^4 cells per microwell. Results are expressed as percentage of specific ^{51}Cr release, calculated by using the formula percentage of specific ^{51}Cr release = $(a - c/b - c) \times 100$, where a = cpm released in the presence of effector cells (splenocytes), b = cpm released by 0.5% Triton × 100 (maximal lysis), and c = cpm released in the control wells without effector cells (spontaneous release). All samples were duplicates. In a different set of experiments mice were injected i.d. with 200 μg of pCMV-NP and sacrificed 4 weeks later. CTL assay was done by following the protocol described above.

Viral Challenge. Mouse-adapted influenza virus A/HK/68 (H3N2) (provided by I. N. Mbawuike, Baylor College of Medicine, Houston) was propagated in the lungs of adult BALB/c mice. For intranasal viral challenge, groups of 10 mice were lightly anesthetized by metofane inhalation (Pitman–Moore, Washington Crossing, NJ) and were inoculated on the nares with 50 μl of a viral suspension containing the previously determined LD_{90} for the virus. Survival was assessed for 25 days after inoculation.

PCR Analyses. BALB/c mice were injected i.d. at the tail base with a single dose of 100 μg of pCMV-NP. Three months later, mice were sacrificed, and their tissues were frozen in liquid nitrogen and stored at −80°C. Total DNA was isolated from the thawed tissues by overnight digestion at 65°C in 0.5 ml of 50 mM Tris, pH 8.0/100 mM EDTA, pH 8.0/100 mM NaCl/1% SDS/proteinase K (0.5 mg/ml). Samples were extracted once with phenol and chloroform, washed with 70% ethanol, dried, and resuspended in 50 μl of TE buffer (10

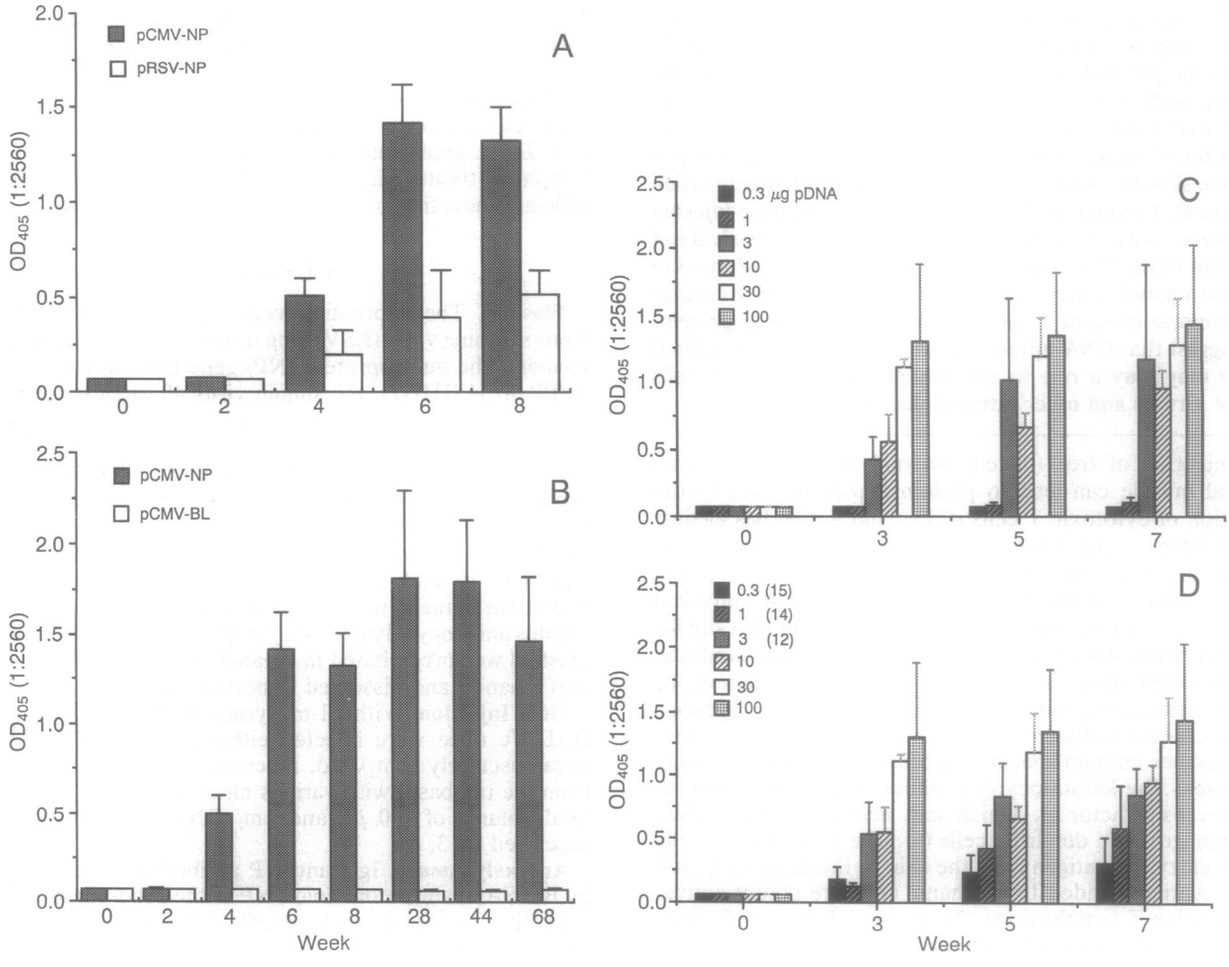

FIG. 1. Antibody response to the influenza NP, after i.d. genetic vaccination in BALB/c mice. IgG anti-NP antibodies were measured by ELISA using recombinant NP as the solid-phase antigen. Serum samples were serially diluted starting at 1:40; OD reading at 1:2560 serum dilution is shown. (*A*). Effect of promoter (CMV or RSV) on the anti-NP IgG response after i.d. gene immunization (n = 4 per group). (*B*) Long-term kinetics of anti-NP IgG response after i.d. immunization with 15 μg of DNA from an NP-expressing vector (pCMV-NP) or a control vector without insert (pCMV-BL) (n = 4 in each group). (*C*) Dose–response relationship between μg of pCMV-NP DNA injected (single injection) and the anti-NP IgG response without DNA carrier (n = 3 per group). (*D*) Dose–response relationship between μg of pCMV-NP DNA injected (single injection) and the anti-NP IgG response, using pUC19 as a DNA carrier. Values in parentheses are the amounts of carrier DNA (μg) mixed with pCMV-NP before a single i.d. injection (n = 3 per group).

mM Tris, pH 7.4/1 mM EDTA). PCR reactions were done on 1 μg of DNA in 10 mM Tris, pH 8.8/5 mM $MgCl_2$/0.001% gelatin/200 mM dNTPs/100 pmol of each primer/2 units of *Taq* polymerase (Stratagene). The primers were designed to produce a 555-nt band from the NP gene of influenza virus strain A/PR/8/34 and had the sequences 5'-ATGGAGAC-TGATGGACAAC-3' (sense strand) and 5'-TGATCATCCT-GACCAATTCCAT-3' (antisense strand). Reactions were run under the following conditions: 1 cycle: 94°C—5 min, 50°C—2 min, 72°C—3 min; 30 cycles: 94°C—1 min, 50°C—2 min, 72°C—3 min; 1 cycle: 94°C—1 min, 50°C—2 min, 72°C—10 min. The DNA products were separated by agarose gel electrophoresis and analyzed by Southern blotting using a 300-bp ^{32}P-labeled internal NP probe.

Immunohistochemical Analysis of Transfected Tissues. Mice were injected i.d. with 100 μg of pCMV-NP or pCMV-BL DNA. Three, ten, and thirty days after injection, the animals were sacrificed. The tails were fixed in 70% cold ethanol for 72 hr and then embedded in paraffin after dehydration. Seven-micrometer tissue sections were prepared and rehydrated by passing through decreased concentrations of ethanol until equilibration with the staining buffer. The slides were incubated for 1 hr at 37°C with 1:100 dilution of goat anti-NP, type A influenza antiserum (National Institutes of Health Research Reference Reagent, National Institute of Allergy and Infectious Diseases, Bethesda, MD), washed extensively with phosphate-buffered saline, and incubated for 1 hr at 37°C with 1:1000 alkaline phosphatase-conjugated mouse anti-goat IgG (Jackson ImmunoResearch). After washing, antibody binding was visualized by development for 15 min at room temperature with an alkaline phosphatase-staining kit using naphthol AS-B as developer (Sigma). The slides were counterstained with eosin and examined by light microscopy.

RESULTS

Antibody Responses. The efficacies of the pCMV-NP and pRSV-NP plasmids for i.d. immunization are compared in Fig. 1*A*. In transient transfection assays, the vectors produced similar amounts of NP protein, and the immune response induced by both plasmids was equivalent after i.m. injection (results not shown). However, the pCMV-NP vector (three injections of 15 μg of DNA at 2-week intervals) stimulated higher antibody responses than did the pRSV-NP vector (under the same conditions) after i.d. immunization (Fig. 1*A*). This may reflect a promoter specificity in the transfected skin cells. Recently, the CMV promoter was shown to express the highest levels of a transgene product in different tissues *in vivo* when compared with other promoters, including RSV (13).

To evaluate the long-term kinetics of anti-NP IgG response, serum samples of i.d.-injected mice (three injections of 15 μg of DNA at 2-week intervals) were analyzed serially for 68 weeks. All animals produced IgG, anti-NP antibodies that peaked initially 6 weeks after pCMV-NP injection and persisted through the 68th week without any drop in titer (Fig. 1*B*).

To determine the minimal amount of pDNA necessary for i.d. vaccination, the animals were given a single injection of different doses of pCMV-NP, and antibody levels were assayed 3, 5, and 7 weeks afterward (Fig. 1*C*). Three micrograms of pDNA was the lowest immunogenic dose. However, if the pCMV-NP was mixed with nonspecific carrier DNA (pUC19), as little as 0.3 μg of pCMV-NP was sufficient to induce an immune response (Fig. 1*D*). Antibody levels increased more quickly in mice injected with more DNA. However, at later time points, antibody titers were nearly equivalent in animals injected with 3–100 μg of pDNA.

To compare the anti-NP antibody response induced by genetic vaccination by the i.d. and the i.m. routes, mice were given a single injection of 1 μg and 10 μg of pCMV-NP i.d. and i.m. Antibody levels were assayed for 10 weeks afterward (Fig. 2). The kinetics of the anti-NP antibody responses after i.d. and i.m. DNA vaccination were different. i.d. DNA vaccination yielded, in general, 4-fold higher antibody titers from the second through the tenth week after vaccination (mean endpoint titer 1:5120) than i.m. injection (mean endpoint titer 1:1280) at equivalent amounts (10 μg) of pDNA.

CTL Responses. To determine if i.d. gene injection could induce a specific and long-lasting CTL response, mice were sacrificed 70 weeks after pCMV-NP injections (three injections of 15 μg of DNA at 2-week intervals), and their splenocytes were used to set up standard mixed lymphocyte cultures in the presence of a synthetic peptide representing the major H-2K^d-restricted CTL epitope on the NP protein (1, 3, 12). The cultures were assayed for anti-NP CTL activity 6 days later using NP peptide-pulsed syngeneic P815 tumor cells as targets. Mixed lymphocyte cultures prepared from these animals displayed high levels of specific anti-NP cytolytic activity, reaching 50%–90% and 70%–100% of specific Cr release at an effector-to-target ratio of 10/1 and 30/1, respectively. The same range of CTL activity was obtained

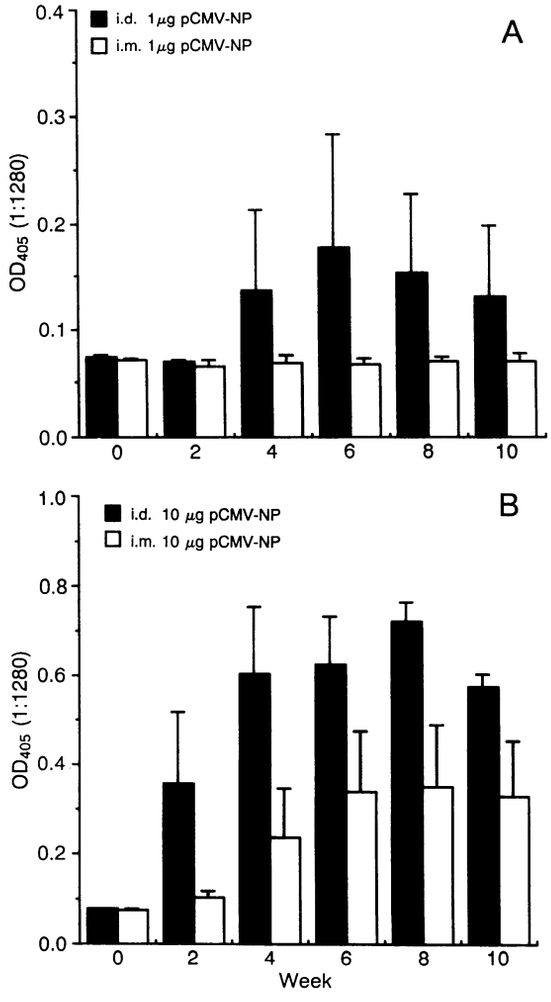

FIG. 2. Comparison between i.d. and i.m. genetic vaccination. Short-term kinetics of anti-NP IgG responses after i.d. and i.m. genetic vaccination. Serum samples were serially diluted starting at 1:40; OD reading at 1:1280 serum dilution is shown. (*A*) Genetic vaccination with single injection of 1 μg of pCMV-NP (n = 3 per group). (*B*) Genetic vaccination with single injection of 10 μg of pCMV-NP (n = 3 per group).

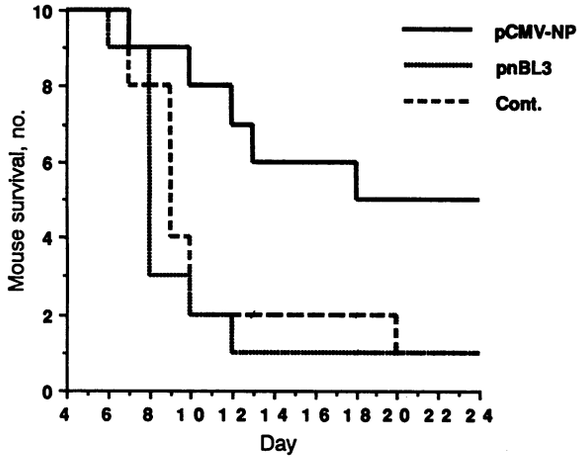

FIG. 3. Survival of i.d. DNA-vaccinated mice after intranasal virus challenge. Mice were immunized i.d. three times with 15 μg of pCMV-NP, with 15 μg of negative control plasmid (pnBL3), or with no plasmid (control; Cont.). Mouse-adapted influenza virus, strain A/HK/68 (H3N2), was inoculated intranasally at a previously determined LD_{90}. Survival was assessed for 25 days after infection (n = 10 per group).

from splenocytes of mice sacrificed 4 weeks after a single i.d. injection of 200 μg of pCMV-NP. Control mice (uninjected animals) gave a maximum of 10% specific lysis.

Protection from Viral Challenge. To determine whether i.d. DNA vaccination could protect animals from viral challenge, mice were injected three times at 2-week intervals with 15 μg of the pCMV-NP plasmid, which contains the NP gene from the A/PR/8/34 (H1N1) strain of influenza virus. Another group of animals were injected with a control plasmid (pnBL3). Two weeks after the last DNA injection, the animals were challenged with a LD_{90} dose of a heterologous influenza strain, A/HK/68 (H3N2). The survival data are shown in Fig. 3. Five of the i.d. vaccinated mice, but only one of the control mice, survived influenza infection ($P < 0.01$). The level of protection after NP gene vaccination tends to vary. The protection seen in Fig. 3 is with the range that we and others (1, 3) have observed with i.m. vaccination.

Gene Expression in Skin. To determine the location of the injected plasmid, Southern blots of PCR-amplified DNA extracted from different tissues were done 3 mo after a single i.d. injection of 100 μg of pCMV-NP. The NP-specific 555-bp fragment was detected at the injection site (tail) but not in other organs (Fig. 4). In other experiments, residual NP DNA could still be amplified from the skin 5 mo after injection.

Immunohistochemical analyses with anti-NP antibody were done 3, 10, and 30 days after a single i.d. injection of 100 μg of pCMV-NP. NP expression was detected in discrete areas of the epidermis and in the upper layers of the dermis (Fig. 5). Positive cells included keratinocytes, fibroblasts, and those with the morphologic appearance of dendritic cells. NP expression in the epidermis and the dermis was greater at 10 days than at 3 days after pDNA injection. Thirty days after pDNA injection, the NP was detected mostly in the dermis; some residual expression remained in the outer epidermal layers. The tail muscles in all the animals and the skin from control tails were completely negative. There were no signs of inflammation in the skin of the pCMV-NP-injected animals. Thus, it appears that antigen is expressed in both the

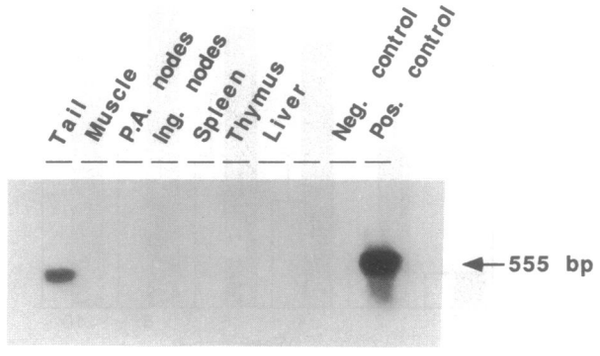

FIG. 4. Southern blot analysis of the NP PCR fragment. The 555-bp NP-DNA fragment was detected in amplified DNA extracted from mouse tails 3 mo after a single injection of 100 μg of pCMV-NP. We did not detect any signal from DNA(s) extracted from other tissues. The pCMV-NP vector served as a positive (Pos.) control, and the pCMV-luciferase vector served as a negative (Neg.) control. P.A., paraaortic lymph nodes; Ing., inguinal lymph nodes.

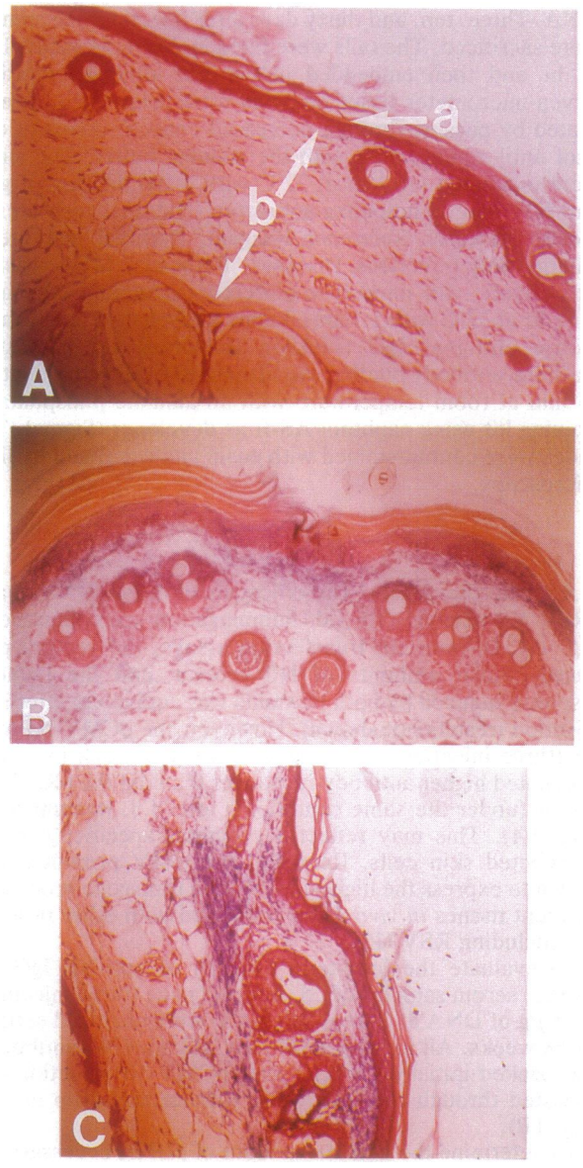

FIG. 5. Immunohistochemical analysis of the transgene product (NP) in pCMV-NP-injected tails. (*A*) The epidermis (*a*) and dermis (*b*) of tail segments taken from a control mice injected with single injection of 100 μg of pCMV-BL. No NP staining was observed in these tail segments 3, 10, and 30 days after pDNA inoculation. (*B*) A tail segment processed 10 days after a single i.d. injection of 100 μg of pCMV-NP showing expression of the NP (purple-blue) in the epidermis and dermis. (×100.) (*C*) A tail segment processed 30 days after a single i.d. injection of 100 μg of pCMV-NP showing a high level of NP expression in the dermis. (×100.)

dermis and epidermis soon after injection and that the epidermal cells that expressed the NP (keratinocytes) move outward as the cells are sloughed during epidermal growth.

DISCUSSION

Our experiments indicate that skin cells exposed *in vivo* to remarkably small amounts of free pDNA can take up the DNA and induce long-lasting cellular and humoral immune responses against the encoded viral protein antigen. A single i.d. injection of <1 μg of specific DNA was sufficient to induce an immune response against the influenza virus NP. The immune mice were partially protected from a lethal challenge with a heterologous influenza strain. Anti-NP antibody titers in the i.d.-vaccinated mice remained high for 68 weeks without any need for boosting. Furthermore, the anti-NP CTL response was also positive 17 mo after i.d. plasmid injection. Such long-lasting cellular and humoral immune responses probably result from continual expression of the antigen and, therefore, continual activation of the immune system.

Although i.d. penetration of NP DNA induced strong immune responses, intrasplenic and intranasal inoculations of the same pDNA preparation were not reproducibly effective (data not shown). Experiments with protein antigens have shown that the density and state of activation of antigen-presenting macrophages and dendritic cells at the injection site strongly influence subsequent immune responses. The abundance of Langerhans' cells in the skin may facilitate the initiation of immune responses, particularly at very low antigen concentrations (8, 14, 15).

Because DNA-transfected cells expressing neoantigens can persist for long periods, they may become targets for immune attack (11). In principle, this can lead to local inflammation and to the cessation of antigen expression. The epidermis not only contains abundant antigen-presenting cells but also expresses high levels of lipocortin, a potent natural inhibitor of inflammation (16). This combination may facilitate the initiation of immune responses by neoantigens, while restraining inflammatory responses. Indeed, leukocytes were not present at the epidermal sites of NP production 3, 10, and 30 days after gene administration.

In the natural state, most foreign antigens are first confronted at the skin or mucosa. In some respects, therefore, gene administration into the superficial skin mimics a normal state. However, it has not been appreciated previously that the cells of the skin can capture and express free pDNA. Indeed, one rationale for the "gene gun," which bombards cells with DNA-coated gold microspheres, is that it overcomes this physical barrier (9, 10, 13, 17). But our results indicate that complicated procedures for i.d. gene vaccination are entirely unnecessary. We have recently observed that abrasion of the skin with a plastic tuberculin-testing device coated with free NP DNA induced a strong immune response (E.R. and D.A.C., unpublished results). It thus seems that i.d. DNA immunization does not require needle injection, as long as the DNA penetrates the cornified epithelium and reaches the skin-associated-lymphoid tissues or other viable skin cells.

Altogether, these results indicate that i.d. DNA immunization elicits strong and persistent humoral and cellular immune responses. The continuous expression of the neoantigen in the skin and its processing and presentation by the skin-associated-lymphoid tissues may reproduce a natural process. This mode of gene delivery resulted in longer lasting immune responses than are usually obtainable by protein vaccines and also induced the formation of cross-reactive CTL. It is well established that the Langerhans' and dendritic cells of the skin are specialized for the uptake and expression of protein antigens. In addition, antigen-presenting cells may be able to take up and express foreign DNA released by dying cells after viral infection. Such a mechanism would facilitate the induction of CTL responses against viruses, especially those that do not directly infect macrophages, dendritic cells, or other antigen-presenting cells, and insure that the major viral antigens enter the class I processing pathway.

It was originally thought that striated muscle cells were unique in their ability to take up and express free DNA *in vivo* (5, 18). However, the intensity of expression of the NP antigen by epidermal and dermal cells exposed to free pDNA belies this interpretation. Instead, the data raise the possibility that the induction of an immune response by exogenous DNA may be a basic biologic phenomenon in the skin and the skin-associated-lymphoid tissues.

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