

The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine

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Background: DNA vaccines reduce IgE responses to selected allergens, but severe reactions to the expressed antigen may limit the usefulness of the technique in allergen immunotherapy.

Objective: We sought to determine the extent of spread of an injected DNA vaccine in mice.

Methods: We placed the gene encoding the potent *Hevea* latex allergen Hev b 5 in a mammalian expression vector and injected this DNA vaccine subcutaneously into BALB/c mice. At several times after injection, the presence of Hev b 5 transcript was determined in multiple tissues by RT-PCR. The identity of the amplification product was confirmed by Southern hybridization and restriction analyses.

Results: Hev b 5 RNA appeared at the injection site and in the lymph nodes, spleen, and lungs within 1 day after injection and persisted for at least 14 days. Hev b 5 RNA was also identified in the blood and tongue 14 days after injection. Antibody and cell-mediated responses to Hev b 5 were also noted in the immunized animals at later time points. As expected, animals injected with the identical plasmid containing the Hev b 5 DNA in the antisense orientation mounted no immune response to Hev b 5.

Conclusions: The rapid and widespread appearance of the Hev b 5 transcript in the injected mice confirms that DNA is translocated from the injection site, transcribed, and expressed in immune and nonimmune tissues after injection. Controlling the extent and degree of expression in specific target tissues may allow therapeutic DNA vaccination with plasmids that encode potentially toxic allergens. (*J Allergy Clin Immunol* 1998;102:469-75.)

Key words: Rodent, allergy, vaccination, immunotherapy, latex

Latex allergy is a severe, untreatable disorder that can lead to disability and death. Because latex allergy is an IgE-mediated disease, it should be amenable to treatment

Abbreviations used

CTL: Cytotoxic T lymphocyte

MBP: Maltose-binding protein

NAL: Nonammoniated latex.

with specific immunotherapy. However, anaphylactic episodes during the course of skin tests have suggested that classical immunotherapy approaches may be associated with an unacceptable incidence of adverse reactions. Indeed, at the present time, only limited specific immunotherapy regimens have been attempted in a small number of patients. One alternative technique is the use of DNA vaccines to decrease specific IgE responses.

A DNA vaccine consists of a deoxynucleotide sequence encoding the target antigen inserted into a mammalian expression vector. After injection, antigen is detected in surrounding muscle fibers within 1 to 2 days,¹⁻³ the plasmid DNA persists for up to 19 months,⁴ and an immune response continues for at least 16 months.⁵ DNA vaccines appear to offer the possibility of long-lasting antigen presentation in the context of a classical MHC restricted immune response without the need for multiple boosting injections or adjuvant vehicles. Cytotoxic T lymphocyte (CTL) activation is a consistent finding,⁵⁻¹² possibly in the context of MHC class I restricted antigen presentation on the surface of transfected muscle fibers.¹³ However, antigen-specific IgG antibody production is observed often as well, presumably after helper T-cell activation, which is an MHC class II restricted process.^{2,5-8,10,11,14-17}

Because recent studies have demonstrated that DNA vaccines could result in a substantial reduction in specific IgE responses to antigens,¹⁸⁻²⁰ we have begun to examine the possibility that DNA vaccines could be used for the immunotherapy of severe latex allergy. In our previous work specific IgE responses to the latex allergen Hev b 5 in alum are decreased by administration of a DNA vaccine expressing Hev b 5 cDNA.²¹ However, because Hev b 5 is highly immunogenic²² and can result in severe localized reactions in presensitized animals (Slater JE, unpublished observations), we propose to control the in vivo expression of Hev b 5 encoded by a DNA vaccine. In doing so, we hope to decrease the adverse antibody-mediated responses to the expressed allergens and increase the likelihood of successful DNA vaccine therapy as an alternative to standard allergen immunotherapy.

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In this study we report that the injection of the plasmid encoding Hev b 5 results in the rapid appearance of the Hev b 5 transcript in the injection site, draining lymph nodes, spleen, blood, and muscle before the appearance of the immune response. Our data provide direct confirmation that under these conditions the transcript, and presumably the injected DNA, appear in both lymphoid and nonlymphoid tissues. Thus the use of promoters that limit the expression of the encoded gene sequence to specific immunologic cell types may offer an opportunity to study and control the immune responses to these antigens.

METHODS

Hev b 5

The sequence cDNA Hev b 5 (839 bp) was cloned and expressed from a *Hevea brasiliensis* latex cDNA library as previously described (GenBank accession number U42640).²² The sequence was expressed in the pMAL/c-2 plasmid (New England Biolabs) as part of a maltose-binding protein (MBP) fusion protein (Hev b 5/MBP). Hev b 5/MBP and MBP were biotinylated by using N-hydroxysuccinimidobiotin as previously described.²³ Nonammoniated latex (NAL) was prepared as previously described.²⁴

Construction of the plasmid

cDNA Hev b 5 was inserted into the expression vector p394 in the sense and antisense orientations, downstream of the human cytomegalovirus major immediate early promoter and upstream of the polyadenylation signal by means of an *EcoRI* cloning site. Plasmids containing either the sense or antisense inserts were identified by restriction enzyme mapping, grown, and purified as previously described.²⁵ The sense plasmid used herein is designated pJES4, and the antisense plasmid is designated pJES2.

pJES2 DNA and pJES4 DNA were prepared by using Qiagen columns or a commercial service (Merlin, Bio 101); the endotoxin content in the DNA preparations used for injection was determined to be less than 125 EU/mL by limulus lysate assay (Bio Whittaker). At most, only 0.05 mL of the DNA preparation, containing no more than 6 EU, was injected into each animal.

Animals

BALB/c mice, 6 to 8 weeks old, were immunized with pJES2 or pJES4 DNA under light anesthesia with methoxyflurane (Pitman-Moore). All injections were at the base of the tail in a volume of 25 μ L of sterile water. For the measurement of immune responses to Hev b 5, mice were injected 3 times at 2-day intervals, with 25 μ g of plasmid DNA per injection. For the detection of Hev b 5 transcripts, only a single 25- μ g injection of plasmid DNA was administered. Serum was obtained by tail bleeds. All procedures were approved by the institutional Animal Research Committee in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International guidelines.

IgG subclass ELISA

Polyvinyl chloride microtiter plates (Falcon Microtest III) were coated with rHev b 5/MBP (10 ng/well) overnight in 0.1 mol/L sodium bicarbonate and blocked with 50 mmol/L Tris (pH 7.4) and 150 mmol/L NaCl containing 0.02% Tween 20 and 1% BSA (dilution buffer). Serum was added in dilution buffer. After overnight incubation, wells were washed and incubated in series with biotinylated anti-mouse IgG1 or biotinylated anti-mouse IgG2a (1:1000 vol/vol, Binding Site) and peroxidase-labeled streptavidin (100 ng/mL, Kirkegaard and Perry). After further washes, the wells were developed with TMB peroxidase substrate (Kirkegaard and Perry),

and the absorbance at 450 nm was determined. With each assay, a standard curve was generated by using serial dilutions of a polyclonal ascites fluid from BALB/c mice immunized with rHev b 5/MBP in Freund's adjuvants. The normalized titer was determined by comparison of the sample absorbance to the standard curve.

For inhibition studies, the serum was preincubated with Hev b 5/MBP for 1.5 hours at room temperature before the addition of the serum to the microtiter wells.

Total IgE ELISA

Polystyrene (Immulon 4) microtiter plates were coated with rat monoclonal anti-mouse IgE (Pharmingen; 100 ng/well) overnight in 0.1 mol/L sodium bicarbonate and blocked with dilution buffer. Serum was added in dilution buffer. After overnight incubation, wells were washed, and biotinylated anti-mouse IgE (1:1000 vol/vol; Binding Site) was added in dilution buffer. After 2 hours of incubation, wells were washed, and peroxidase-labeled streptavidin (Kirkegaard and Perry; 100 ng/well) was added in dilution buffer. The wells were incubated for 1 hour more, washed, developed with TMB substrate, and read at 450 nm. The results were compared with a standard curve generated with monoclonal mouse IgE (Pharmingen).

Antigen-specific IgE ELISA

IgE specific for rHev b 5 and for MBP were determined in assays identical to the total IgE ELISA except that biotinylated rHev b 5/MBP or biotinylated MBP (100 ng/well) were added instead of biotinylated anti-IgE. With each assay, a standard curve was generated by using serial dilutions of a polyclonal ascites fluid generated from BALB/c mice immunized with rHev b 5/MBP in alum. The normalized titer was determined by comparison of the sample absorbance to the standard curve.

Splenocyte culture

Mice were killed by means of cervical dislocation, and their spleens were removed aseptically 90 days after initial immunization. Splenocytes were prepared, and erythrocytes were removed by hypotonic lysis. Cells (10^5) were incubated for 1, 4, and 6 days with Hev b 5/MBP or MBP in serum-free culture medium (Aim V, Life Technologies) supplemented with 50 μ mol/L 2-mercaptoethanol.

Cell proliferation was estimated at 4 days by means of a colorimetric assay. Thiazolyl blue (MTT) (Sigma) was added to the culture medium to a final concentration of 0.5 mg/mL. After incubation for 4 hours at 37° C, cells were lysed overnight by addition of 0.25 to 1 volume of 10% SDS in 0.01N HCl. The absorbance of the formazan cleavage product was measured at 570 nm, with a reference wavelength of 650 nm. Cell number was then estimated by comparison with a standard curve of precounted splenocytes grown in media at the same time.

Cytokine assays

IL-4 and IFN- γ were measured by sandwich ELISA. Capture antibodies (Pharmingen 18031D and 18181D) were bound to polystyrene (Immulon 4) microtiter plates in 0.1 mol/L sodium bicarbonate, and the wells were blocked with dilution buffer. Culture supernatants were added, and bound cytokine was detected with biotinylated antibody (Pharmingen 18042D and 18112D) and peroxidase-labeled streptavidin (Kirkegaard and Perry; 100 ng/well). The wells were incubated for 1 hour more, washed, developed with TMB substrate, and read at 450 nm. The results were compared with a standard curve generated with pure recombinant standards (Pharmingen).

RT-PCR

Mice that had been injected with pJES4 were killed by means of cervical dislocation at various time points after injection. Tails,

spleens, lungs, tongues, and superficial inguinal lymph nodes were quickly excised and frozen by immersion in liquid nitrogen. Blood was collected postmortem and frozen. RNA was isolated from frozen tissue by using RNeasy B (Tel-Test, Inc), according to the manufacturer's protocol. Nucleic acid yield and quality were estimated by UV absorption at 260 nm and 280 nm. First strand cDNA was generated from 1 μ g of RNA by using Superscript RT or M-MLV RT (Life Technologies), according to the manufacturer's directions, with an oligo-dT primer. PCR was performed on the cDNA by using Hev b 5-specific primers, which correspond to nucleotides 90 to 109 and 341 to 363 of the Hev b 5 cDNA sequence.²² The PCR reaction mixtures contained 10 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 0.001% gelatin, dNTP (200 μ mol/L each), primers (500 nmol/L each), 2.5 U of Taq DNA polymerase (Promega), and the templates in a total volume of 50 μ L. The Taq DNA polymerase was added to the reaction mixtures after an initial denaturation step of 94° C for 5 minutes. The mixtures were then subjected to 40 amplification cycles as follows: 94° C for 45 seconds, 55° C for 25 seconds, and 72° C for 3 minutes. At the end of 40 cycles, a final extension step at 72° C was performed for 25 minutes. In all experiments cDNA preparations from tissues of uninjected BALB/c mice were amplified as controls. cDNA samples were also amplified with β -actin specific primers.

Southern blot

PCR-amplified cDNA was separated by electrophoresis in a 2% agarose gel in TBE and transferred to a GeneScreen Plus membrane (Dupont/NEN). The membrane was then probed overnight at 65° C with plasmid containing the Hev b 5 sequence that had been ³²P-labeled by the random primer labeling technique by means of exo(-) Klenow enzyme and [α -³²P]dCTP (Prime-It II, Stratagene). After repeated high stringency washes, the membrane was exposed to X-omat film at -70° C.

Restriction analysis

Amplified Hev b 5 cDNA was separated by agarose electrophoresis, removed from the agarose by filtration centrifugation (Ultrafree-MC, Millipore), and purified by ethanol precipitation. The fragment was then digested with *Sfa*NI (New England Biolabs) and analyzed by agarose electrophoresis.

Statistical analyses

The significance of the observed changes in antibody titer and cell proliferation were estimated by a two-tailed *t* test.

RESULTS

BALB/c mice injected with pJES4 mount an immune response to the encoded antigen, Hev b 5

No adverse reactions were noted in naive mice injected in the tail with pJES2 or pJES4. Mice injected with pJES4, but not pJES2, developed detectable IgM, IgG1, and IgG2a responses to the encoded antigen Hev b 5. As expected, the IgM response was transient, appearing by 25 days after injection and no longer present at 55 days. The IgG responses appeared at 55 days after injection and persisted over the remainder of the period of observation (100 days). In all cases the responses were 5- to 10-fold lower than those observed when BALB/c mice were immunized with protein antigen in Freund's adjuvants,²² as indicated by the maximal normalized titers (Fig. 1). No changes in specific IgE or total IgE were

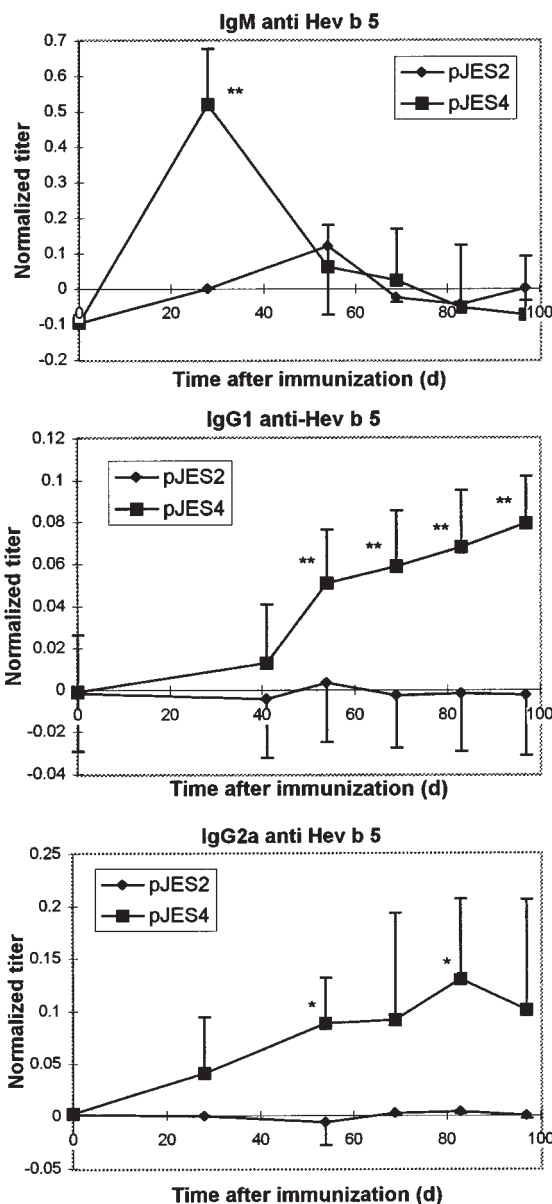


FIG 1. Hev b 5-specific antibody responses to injection with pJES4 (Hev b 5-sense) and pJES2 (Hev b 5-antisense). Mice were tail bled at indicated times after initial injection. Serum was diluted and applied to microtiter wells that had been coated with Hev b 5/MBP (10 ng/well) and blocked. IgM antibody was detected by incubating wells with peroxidase-labeled rabbit-anti-mouse IgM; IgG1 and IgG2a were detected by incubating, in sequence, with biotinylated rabbit anti-subtype antibody and peroxidase-labeled streptavidin. Color was then developed with TMB substrate. Each data point represents average of 3 mouse sera $\pm$ SD. Significance was determined by *t* test. **P* < .05; ***P* < .005.

detected, and the specific IgG1 and IgG2a responses were completely inhibited by 1 μ g/mL of Hev b 5/MBP (not shown).

Splenocytes from pJES4-sensitized mice responded to culture in the presence of Hev b 5/MBP with increased proliferation (Fig. 2). Release of IFN- γ into the culture fluid was noted when the cells were incubated in the

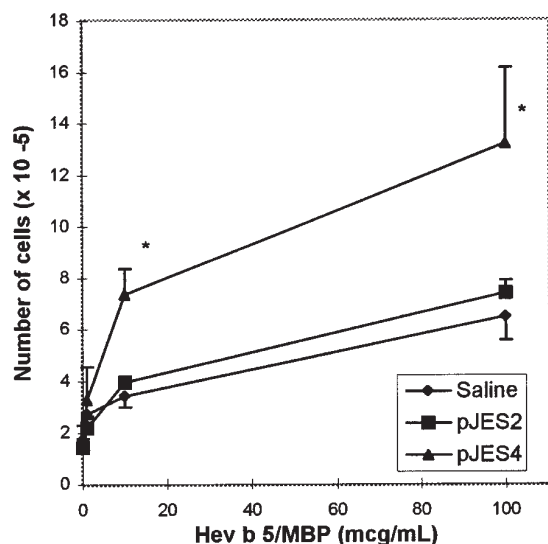


FIG 2. Proliferation of splenocytes from mice injected with saline, pJES2, or pJES4. Cells were cultured for 4 days in presence of 0, 1, 10, or 100 μ g/mL or Hev b 5/MBP. Proliferation was estimated by oxidation of MTT and comparison to standard curve of proliferating cells. Significance was determined by *t* test. **P* < .05.

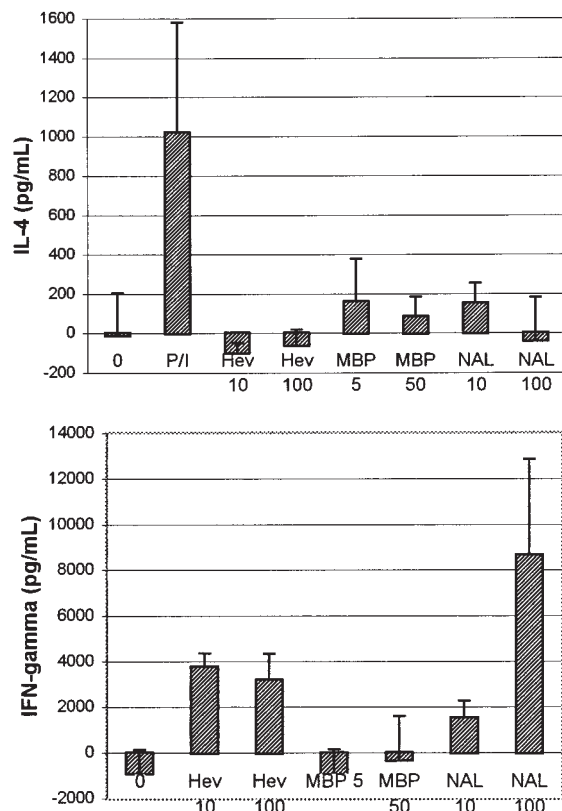


FIG 3. IFN- γ and IL-4 release by splenocytes from mice injected with pJES4. Cytokine release was determined after 1 (IL-4) or 6 (IFN- γ) days of culture in presence of Hev b 5/MBP (10 and 100 μ g/mL), MBP (5 and 50 μ g/mL), and NAL (10 and 100 μ g/mL) or phorbol myristate acetate/ionomycin. Cells from mice injected with pJES2 and from naive mice did not release cytokines to any of the protein antigens (not shown).

presence of NAL and Hev b 5/MBP, but not MBP. Although IL-4 was released from the positive control splenocytes, none was detected from the splenocytes of pJES4-sensitized animals after incubation in the presence of NAL, Hev b 5/MBP, or MBP (Fig. 3).

Hev b 5 RNA is detected at the injection sites, lymph nodes, and spleens of injected mice

We extracted RNA from the tissues of the pJES4-injected mice and analyzed these for Hev b 5 message by means of RT-PCR. A specific and unique band appeared at the predicted length of 274 bp in the amplification products of cDNA from the tails, lymph nodes, spleens, and lungs of the injected mice 1, 3, and 14 days after injection. In addition, Hev b 5 message was detected in the tongue and blood of mice killed 14 days after injection. No amplification products were observed in the tissues of uninjected mice (Fig. 4). Moreover, the absence of contaminating Hev b 5 DNA was verified by the amplification of samples to which no RT was added (data not shown).

The specificity of this band for the Hev b 5 sequence was verified by Southern hybridization (Fig. 5); the unique PCR fragment hybridized specifically to the Hev b 5 probe. No hybridization was observed to the control PCR reaction products or to the genomic mouse DNA digest. To document further the identity of the PCR product, the amplified DNA fragments were cleaved with *Sfa*NI, an enzyme predicted to generate 2 fragments of 155 bp and 119 bp from the PCR product. Indeed, cleavage products corresponding closely to the predicted fragments were observed after digestion with *Sfa*NI (Fig. 6).

DISCUSSION

Latex allergy is a common severe allergic disease for which no treatment is currently available. DNA vaccines have been shown to reduce specific IgE responses in mice and rats and therefore may be of benefit to patients with severe IgE-mediated disease. In our earlier animal studies, however, some presensitized animals treated with a DNA vaccine encoding the latex allergen Hev b 5 experienced severe local inflammatory responses. Therefore we have developed the tools to monitor the transcription of the Hev b 5 gene in the tissues of mice after injection with the DNA vaccine for Hev b 5. Our ultimate goal is to limit the expression of the Hev b 5 gene to specific antigen-presenting cells and therefore to control toxicity.

The mechanism of antigen processing and presentation after DNA vaccination is uncertain. Antigen could be released from the cells after injury by CTLs, which occurs within 2 weeks of injection,¹³ or by active secretion from the cell surface. Cytolysis alone is unlikely to release antigen for MHC class II presentation; expression of luciferase, a nonsecreted protein, results in no detectable IgG responses.²⁶ In one study a T_H1 lymphokine pattern, as indicated by the production of IL-2 but not IL-4, was documented in response to a DNA vaccine to rabies virus glycoprotein.¹⁰ In another study

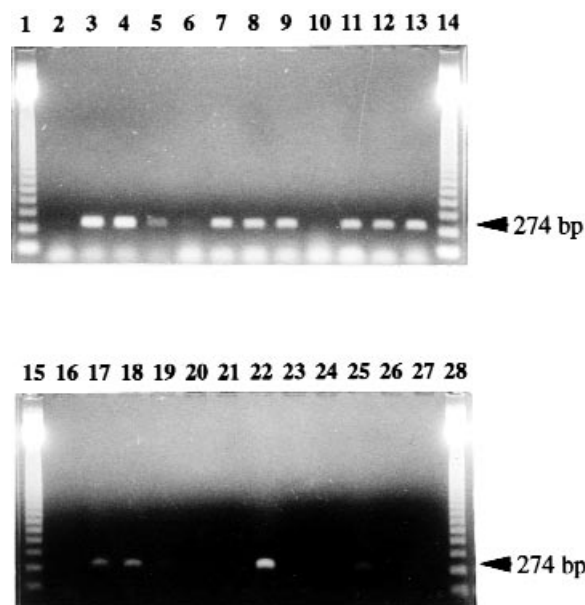


FIG 4. Hev b 5 transcript in tissues of mice injected with pJES4. RNA was extracted from tissues of naive mice and from tissues of mice injected with pJES4 after time intervals of 1, 3, and 14 days after injection. RT-PCR was performed, and amplification products were analyzed by agarose gel electrophoresis. One hundred twenty-three base pair standards (Life Technologies) are shown in lanes 1 and 14. In the top panel, tissues from negative control mice are shown in lanes 2, 6, and 10; tissues from mice injected 1 day before death are shown in lanes 3, 7, and 11; tissues from mice injected 3 days before death are shown in lanes 4, 8, and 12; and tissues from mice injected 14 days before death are shown in lanes 5, 9, and 13. RT-PCR products from the tail are shown in lanes 2 to 5, from the lymph nodes in lanes 6 to 9, and from the spleen in lanes 10 to 13. In the bottom panel, RT-PCR products from the lung are shown in lanes 16 to 19 (control, 1-day, 3-day, and 14-day specimens, respectively). Fourteen-day specimens representing blood (lane 22) and tongue (lane 25) are also shown on the bottom panel. The amplification products are consistent with the predicted length of 274 bp. β -Actin control amplification bands (not shown) were present in all amplified samples but were decreased in lanes 6 and 13.

IFN- γ levels correlated well with CTL activity,⁷ but IL-4 activity has been observed as well.^{7,16} More recent work suggests that the cytokine pattern of the responding lymphocytes is dependent on the route of injection. When the plasmid was injected in the footpad or in the base of the tail, a T_{H1} response developed, whereas intracutaneous delivery of the same plasmid with a gene gun resulted in a T_{H2} response.²⁷

Possible modes of expression control include placing the encoded gene under the control of weaker, tissue-specific, or repressible promoters. As an essential first step in this study, we monitored the transcription of the encoded gene at the injected sites and in lymphoid tissue. In previous work, Raz et al.⁵ attempted, although without success, to detect target DNA sequences 3 months after injection in the spleens and draining lymphoid tissue of DNA vaccine-injected animals. Condon et al.²⁸ were able

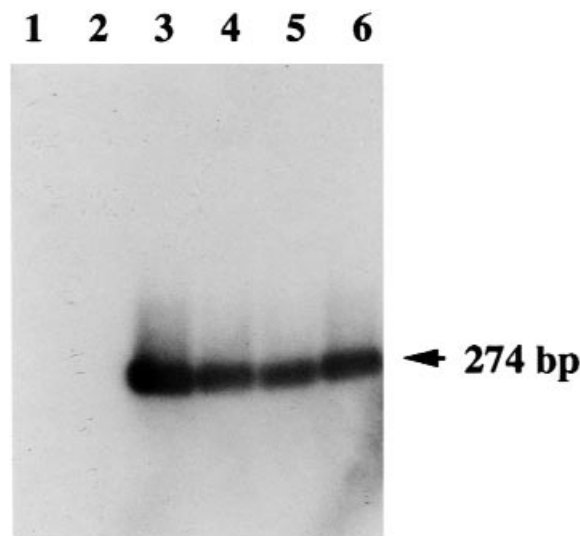


FIG 5. Southern hybridization analysis of Hev b 5 RT-PCR products. PCR-amplified cDNA was separated by electrophoresis on 2% agarose gel, transferred to GeneScreen Plus membrane (Dupont/NEN), and probed with ³²P-labeled cDNA Hev b 5 plasmid. Lanes 1 and 2 contain negative control DNA samples (lambda DNA amplification product and mouse genomic DNA *Eco*RI digest, respectively). Lanes 3 to 6 contain amplification products from the tail, lymph nodes, spleen, and lungs of a mouse killed 3 days after injection with pJES4.

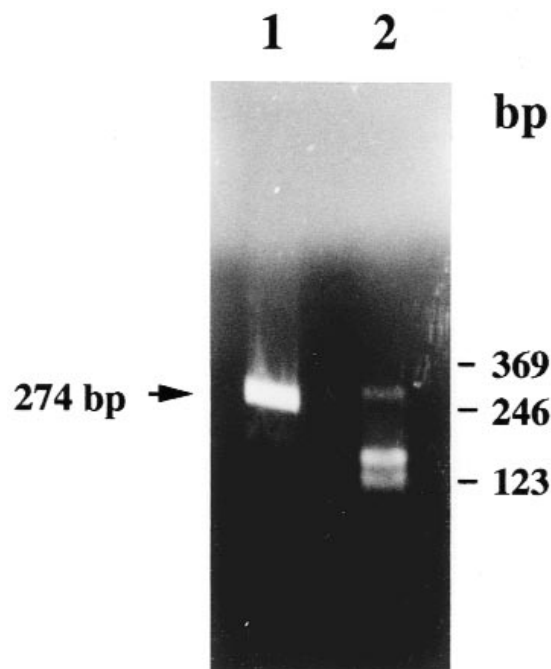


FIG 6. Restriction analysis of Hev b 5 RT-PCR products. Amplified Hev b 5 cDNA was separated by agarose gel electrophoresis (Nusieve), removed from agarose by filtration centrifugation (Ultra-free-MC, Millipore), and purified by ethanol precipitation. The fragment was then digested with *Sfa*N I (New England Biolabs) and analyzed by agarose electrophoresis. Uncut DNA appears in lane 1, and *Sfa*N I digest is in lane 2. The predicted length of the uncut amplification product is 274 bp; the *Sfa*N I digestion products would be predicted to be 155 and 119 bp.

to demonstrate the presence of the encoded protein of a DNA vaccine in the draining lymph nodes, but did not experimentally verify the presence of the transcript in the same tissue. Casares et al.²⁹ demonstrated that dendritic cells isolated from the skin and draining lymph nodes of a site injected with a DNA vaccine could present the encoded antigen in culture to T cells.

As documented in previous studies with other antigens, pJES4 injection leads to a transient IgM response to Hev b 5 and a more persistent IgG2a response that was somewhat larger than the IgG1 response. No IgE response to Hev b 5 was detected. Splenocytes from mice injected with pJES4 proliferated and released IFN- γ , but not IL-4, in response to Hev b 5. This is consistent with the studies of Felquate et al.,²⁷ in which BALB/c mice had a predominantly T_{H1}-type response to injection of a DNA vaccine at the base of the tail or in the footpad.

Mice injected with pJES4 had rapid and persistent evidence of Hev b 5 transcript at the injection site, as well as in draining lymphoid tissue. The identity of the Hev b 5 transcript was confirmed both by Southern hybridization and restriction analysis of the RT-PCR product. To our surprise, transcript was also detected in the blood, in the tongue, and to a lesser degree in the lungs. This differs from the observation of Raz et al.⁵ that no DNA signal was detected anywhere other than at the injection site. The techniques used in that study (Southern hybridization of an amplification DNA fragment) should be comparable in sensitivity to the RT-PCR technique used here. However, in that study the investigators only looked at the tissues harvested 3 months after injection. In doing so, they may have missed the more widespread presence of the plasmid DNA at earlier time points.

In contrast to other studies, we have not demonstrated the presence of Hev b 5 protein in the tissues. In part this is due to the inadequate sensitivity of our current assays. Although we have been able to detect nanogram quantities of Hev b 5 with inhibition techniques,²² we have been able to measure only microgram quantities with direct immunodetection methods (Slater, JE, unpublished observation). However, it is unlikely that cellular transcript appears without consequent protein expression, especially in the presence of a definite anti-Hev b 5 immune response. Furthermore, the widespread appearance of the Hev b 5 transcript, more so than Hev b 5 protein, is strong evidence of the circulation of the DNA vaccine itself through immune and nonimmune tissues.

This metastasis of the transcript may be due to uptake of the expression vector by local dendritic cells^{28,29} or other circulating antigen-presenting cells. Such intense local and widespread expression may be pathogenic in animals or patients with a preexisting vigorous IgE response to the encoded antigen and may not be necessary for successful immunomodulation. Thus our current studies are aimed at controlling expression by placing the Hev b 5 gene under the control of weak but highly regulated tissue-specific promoters. Such targeted and attenuated expression may be sufficient to effect the desired immunomodulation without adverse reactions.

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