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DNA immunization using a non-viral promoter

T. Vanniasinkam [a], S.T. Reddy [b], H.C.J. Ertl [a,*]

[a] The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA
[b] Department of Pathology, Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, VA 23298-0662, USA

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Abstract

Most DNA vaccines rely on strong viral promoters to optimize levels of transgene expression. Some studies have demonstrated that the potency of viral promoters does not necessarily correlate with DNA vaccine efficacy in vivo. This has partly been attributed to downregulation of these promoters by cytokines such as interferon γ induced by the CpG motives of these vaccines. In an attempt to avoid downregulation of viral promoters by IFN-γ, we tested vaccine vectors driven by the MHC class II promoter. To enhance the activity of this promoter, another plasmid expressing the human MHC class II transactivator driven by a viral promoter, the native IFN-γ inducible CIITA type IV promoter (PIV) or a synthetic promoter containing IFN-γ inducible elements was co-inoculated. Our data show that a non-viral promoter such as the MHC class II promoter tested in this study can indeed be used in DNA vaccines.

Keywords: DNA vaccine; IFN-γ; Rabies; HIV

Introduction

Over the past decade, plasmid DNA has been used extensively for gene transfer into mammalian cells. When used as vaccines in small experimental animals, plasmid DNA induces protective T- and B-cell-mediated immune responses against a range of pathogens including viruses (Whalen et al., 1995; Xiang et al., 1994). Nevertheless, DNA vaccines thus far have performed poorly in clinical trials. The magnitude of immune responses generated by a vaccine in part depends on the antigenic load. Levels of antigen expression by genetic vaccines, such as plasmid vectors, are dictated by the strength of transcriptional regulatory elements and by the transduction efficiency of the plasmid. Most DNA vaccines use strong viral promoters to optimize transgene product expression (Norman et al., 1997; Oran and Robinson, 2003; Xiang et al., 1994). Induction of primary adaptive immune responses does not solely depend on foreign antigen but also requires the antigen to be presented in lymphatic tissues by mature dendritic cells expressing co-stimulatory molecules. Immature dendritic cells are dispersed throughout an organism where they can be transduced by plasmid vectors. The bacterial DNA sequences within a plasmid vector carry unmethylated CpG sequences, which are rare or methylated in mammalian DNA. These sequences are recognized by pattern recognition receptors of the Toll-like receptor (TLR) family, i.e., TLR-9 expressed by cells of the innate immune system as pathogen-associated molecular patterns (Agrawal and Kandimalla, 2003). Interaction of unmethylated CpG sequences with TLR-9 triggers an inflammatory reaction, which results in the release of pro-inflammatory cytokines, such as interferons (IFN), IL-12, IL-6 and TNF-α, and in maturation of dendritic cells (Hemmi et al., 2000). Presence of such motifs is essential for the immunogenicity of DNA vaccines, and elimination or methylation of CpG motifs prevents DNA vaccine-induced activation of adaptive immune responses (Pasquini et al., 1999; Reyes-Sandoval and Ertl, 2004).

IFN released rapidly in response to bacterial DNA by the innate immune system has antiviral activity. IFN-γ enables cells to resist viral replication, in part by inhibiting transcription of viral genes. IFN-γ down-modulates propagation of viruses by a number of pathways such as by the IFN-γ-induced production of transcriptional repressors, which bind to IFN responsive enhancer elements within viral promoters (Darnell et al., 1994; Gribaudo et al., 1993). Thus, although CpG-induced inflammatory responses are essential to drive an adaptive immune response to DNA vaccines, the resulting cytokines may dampen

* Corresponding author. Fax: +1 215 898 3953.
E-mail address: ertl@wistar.upenn.edu (H.C.J. Ertl).

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the production of antigen and thus reduce stimulation of T and B cells as was shown previously, where under certain experimental conditions increase of IFN-γ production reduced the immune response to a DNA vaccine (Xiang and Ertl, 1995).

To test if vectors based on promoters that are rendered more active in presence of IFN-γ can provide an alternative to currently used DNA vaccines based on strong viral promoters, we developed DNA vaccines containing the MHC class II promoter. The MHC class II promoter requires induction by the MHC class II transactivator (CIITA), which can be upregulated by IFN-γ. Here, we show that DNA vaccines encoding the rabies virus glycoprotein or a truncated codon-optimized form of the HIV-1 gag protein under the control of the MHC class II Ea promoter combined with vectors encoding CIITA induce robust transgene product-specific adaptive immune responses in mice, which are increased in the presence of IFN-γ.

Results

Transgene product expression by vectors

DNA vaccine vectors that were used in this study are listed in Table 1. The pCII vectors had been generated previously by replacing the SV40 promoter of the commercially available pSG5 vector with the MHC class II E2a promoter. In the pPIV.CIITA vector, expression of CIITA is driven by the CIITA type IV promoter known to be upregulated by IFN-γ. In the pRep4.CIITA vector, expression is driven by the RSV promoter, which is downregulated by IFN-γ. The promoter controlling expression in the pEn.CIITA vector was designed by us to contain IFN-γ-responsive elements which should result in enhanced promoter activity in presence of this cytokine. PCR (following conversion of mRNA to cDNA) was performed to confirm expression of CIITA in L929 cells transfected with pRep4.CIITA, pPIV.CIITA or pEn.CIITA vectors. Briefly, 48 h following transfection of L929 cells with these plasmids, RNA extraction was performed on the transfected cells using Tri Reagent (Sigma, MO), and samples were then subjected to DNase treatment to remove contaminating DNA prior to cDNA synthesis using M-MLV reverse transcriptase (Invitrogen, CA). CIITA cDNA was detected by RT-PCR using previously described primers (Pai et al., 2002) (results not shown). Plasmid pCIIrab.gp has been tested for in vitro expression previously (Xiang et al., 1997). To confirm gag37 expression by pCII.gag37, NIH 3T3 cells were transfected with this plasmid, and gag37 protein expression was detected by Western Blot using previously described methods (Pinto et al., 2003) (results not shown).

Table 1
List of plasmid vaccines used in this study

Plasmid	Promoter	Transgene
pSG5rab.gp	SV40	Rabies glycoprotein
pCIIrab.gp	MHC class II	Rabies glycoprotein
pRep4.CIITA	RSV	Class II transactivator
pEn.CIITA	Synthetic promoter containing IFN-γ inducible elements	Class II transactivator
pPIV.CIITA	CIITA promoter IV	Class II transactivator
pCII.gag37	MHC class II	Truncated HIV-1 gag

Effect of IFN-γ on viral promoter function

Cell lysates from L929 cells transfected with pSG5LacZ vector with and without IFN-γ treatment were subjected to a β-galactosidase assay (Fig. 1). Results from this assay clearly demonstrated that the activity of the SV40 promoter in pSG5LacZ was significantly compromised in the presence of IFN-γ; with increasing doses of IFN-γ, there was a corresponding decrease in the level of β-galactosidase expression. Comparable results were obtained with vectors expressing the rabies virus glycoprotein (data not shown).

Immune responses to rabies glycoprotein

Vectors were initially analyzed for induction of transgene product-specific B cell responses. Groups of C3H/He mice (5 per group) were injected into the quadriceps muscles with pCIIrab.gp combined with pEN.CIITA, pPIV.CIITA, pRep4.CIITA or as a control with an empty vector. Additional control mice were injected only with the pEn.CIITA vector and an empty vector. Animals were bled 2, 4 and 6 weeks post-immunization, and antibody titers were determined by an ELISA. None of the groups had detectable antibodies to rabies virus by 2 weeks after vaccination (not shown). By 4 weeks following immunization, mice injected with both pCIIrab.gp + pEn.CIITA vectors or pCIIrab.gp + pRep4.CIITA vectors mounted a vigorous antibody response. Mice vaccinated with either pCIIrab.gp vector alone or pCIIrab.gp + pPIV.CIITA vectors had only marginal titers of rabies virus-specific antibodies by week 4 which slightly increased by week 6 in mice that received the pPIV.CIITA + pCIIrab.gp vectors (Fig. 2). The experiment was repeated by assessing antibody titers by a neutralization assay (Table 2). In this experiment, a vector expressing the rabies virus glycoprotein under the control of the SV40 promoter was included. This vector has been studied extensively in comparison to other vectors with different regulatory sequences or different CpG contents and consistently outperformed other DNA vaccines to the rabies virus glycoprotein. Rabies virus neutralizing antibody titers were measured against a reference serum from the World Health Organization to allow for expression of titers in International Units, which can be used as a surrogate measure for protective immunity; titers of or above 0.5 IU are known to protect against a peripheral infection with an otherwise lethal dose of rabies virus. Co-injection of pCIIrab.gp + pRep4.CIITA vectors resulted in antibody titers above 0.5 IU by week 2, which increased further by week 4. Co-injection of pCIIrab.gp + pEn.CIITA vectors achieved protective titers of antibodies to rabies by week 4. Injection of pCIIrab.gp or pCIIrab.gp + pPIV.CIITA vectors failed to induce neutralizing antibody titers of or above 0.5 IU. This indicates that the transactivator is essential to optimize stimulation of antibody responses to the DNA vaccine containing the MHC class II promoter. Results furthermore show that the CIITA promoter IV is not as

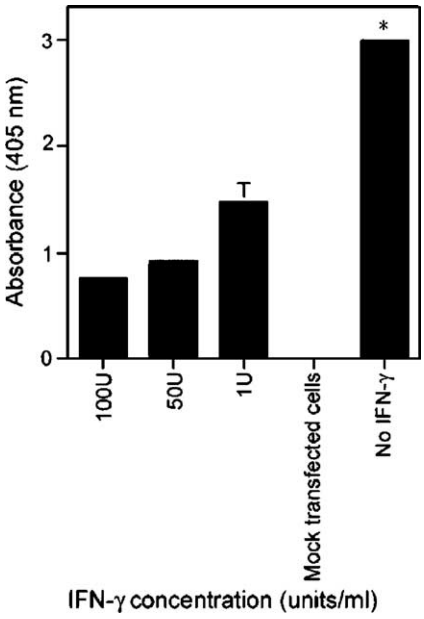

Fig. 1. Effect of IFN-γ on function of viral promoters. L929 cells transfected with plasmid pSG5LacZ were treated with 100 U/ml, 50 U/ml or 1 U/ml of IFN-γ. Following a 48 h incubation, lysates were obtained from these cells and a β-galactosidase assay performed. Data are shown as mean ± SD for triplicate samples. With increasing amounts of IFN-γ, less LacZ expression was detected. The highest expression levels were detected in the samples that had been transfected with pSG5LacZ without IFN-γ treatment (*the absorbance of these samples were greater than 3 and consequently too high to be measured using this spectrophotometer).

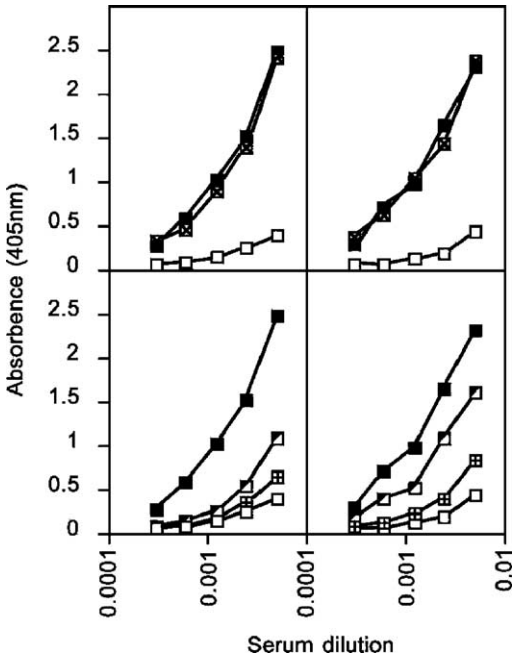

Fig. 2. Rabies virus glycoprotein-specific antibody response at 4 and 6 weeks following a single administration of DNA vaccine. Groups of C3H/He mice were immunized with pCIIrab.gp vector alone (hatched squares), pCIIrab.gp and pEN.CIITA vectors (solid squares), pCIIrab.gp and pPIV.CIITA vectors (half shaded squares), pCIIrab.gp and pRep4.CIITA vectors (squares with a cross) or pEn.CIITA vector only (open squares). Data are shown as mean ± SD for triplicate samples. Data were analyzed using a Student t test. The x and y axes of each graph were normalized to the values/parameters shown to the left and at the bottom. The antibody response in mice immunized with the pCIIrab.gp and pEn.CIITA vector regimen was significantly higher than that obtained with the other vaccination regimens ($P \leq 0.05$) except for both pCIIrab.gp and pRep4.CIITA vectors. There was no significant difference between the mice immunized with pCIIrab.gp and either pEn.CIITA vector or pRep4.CIITA vector.

effective as the RSV promoter in the pRep4.CIITA vector or the synthetic promoter pEn in achieving significant levels of CIITA expression. Near equal performance of the CIITA vectors containing the IFN-inducible artificial promoter or the RSV promoter (the latter which would be downregulated by IFN-γ) may reflect differences in the relative strengths of these regulatory elements that are equalized in the presence of IFN-γ. Alternatively, even small amounts of CIITA may suffice to saturate the system and achieve maximal activation of the MHC class II promoter. The pSG5rab.gp vector induced the highest antibody response.

T cell responses assessed by measuring cytokine release from in vitro stimulated splenocytes mirrored antibody responses, although the response to the pSG5rab.gp vector was only marginally higher that the response to the pCIIrab.gp + pEn.CIITA or pCIIrab.gp + pPREP4.CIITA vectors. Splenocytes of mice immunized with both pCIIrab.gp and pEn.CIITA or pRep4.CIITA secreted significant and comparative levels of IL-2/IL-4 and IFN-γ in response to rabies virus, while cytokine production from splenocytes of mice immunized with pCIIrab.gp + pEn.CIITA vectors or pCIIrab.gp + pRep4.CIITA vectors secreted detectable but significantly lower levels of cytokines (Fig. 3). Splenocytes of mice immunized with pSG5rab.gp secreted slightly higher levels of IFN-γ compared with all other groups, while proliferative responses were comparable. To assess if indeed the artificial promoter pEn was influenced by IFN-γ in vivo, we immunized groups of IFN-gamma knockout (GKO) mice and control C57BL/6 mice with pCIIrab.gp and pEn.CIITA or pEn.CIITA (Fig. 4A). Not surprisingly, GKO mice immunized with pCIIrab.gp + pEn.CIITA vectors exhibited a lower antibody response than the control mice immunized with the same vaccines. Importantly, there was no significant difference in the immune response between GKO mice immunized with both pCIIrab.gp + pEn.CIITA vectors, or pCIIrab.gp + pEn.CIITA vectors, confirming that, in the absence of IFN-γ, the function of promoter pEn and consequently the performance of the MHC class II promoter-driven pCIIrab.gp vector is severely compromised. The pSG5rab.gp vector induced higher antibody

Table 2
Virus neutralizing antibody (VNA) titers to rabies virus determined at 2, 4 and 8 weeks following immunization with various vaccine constructs

Vaccine	VNA titer (2–8 weeks following immunization) (IU/ml)		
	2 weeks	4 weeks	8 weeks
pCIIrab.gp	<0.5	<0.5	<0.5
pEn.CIITA	<0.5	<0.5	<0.5
pSG5rab.gp	4	20	16
pEn.CIITA + pCIIrab.gp	<0.5	3	6
pPIV.CIITA + pCIIrab.gp	<0.5	<0.5	<0.5
pRep4.CIITA + pCIIrab.gp	2	6	4

VNA titers over 0.5 IU confer protection against rabies virus infection.

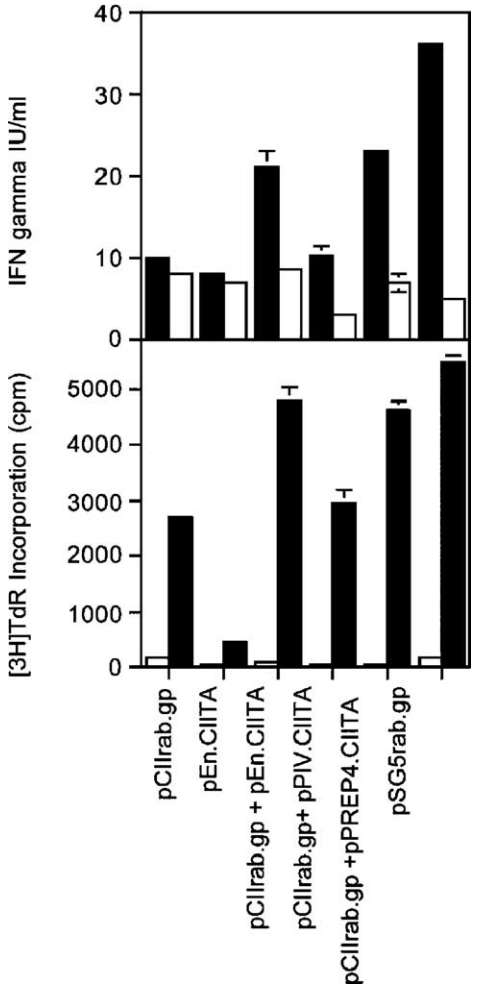

Fig. 3. Cytokine release from splenocytes 6 weeks following immunization with various plasmids. Splenocytes were incubated with 1 μg ERA-BPL (black bars) or media (white bars) for 48 h and the supernatants obtained for cytokine testing. Top graph: supernatants were tested for the presence of IFN-γ. Bottom graph: supernatants were tested for induction of HT-2 cell proliferation (IL-2/IL-4 detection). Mice immunized with pSG5rab.gp vector demonstrated the highest immune response. However, mice immunized with pEn.CIITA + pCIIrab.gp vectors had a significantly higher IL-2/IL-4 and IFN-γ response than mice immunized with pEn.CIITA vector only, pCIIrab.gp vector only or both pEn.CIITA and pPIV.CIITA vector ($P \leq 0.05$). There was no significant difference in the cytokine responses between mice immunized with pCIIrab.gp vector and either pEn.CIITA or pRep4.CIITA vectors.

responses in GKO mice than in C57Bl/6 mice (Fig. 4B), indicating that under certain experimental conditions IFN-γ indeed can dampen the antibody response to a DNA vaccine's transgene product controlled by a viral promoter.

Levels of expression of transcripts in vivo

To further evaluate the effect of IFN-γ on antigen production by the CIITA-induced MHC class II promoter, we determined copy numbers of vector and vector-derived transcripts in muscles and lymph nodes of C57BL/6 and GKO mice injected with pCIIrab.gp alone or with pCIIrab.gp + pEN.CIITA. Groups of 5 mice were injected with the various vectors, and the injected muscles and their draining lymph

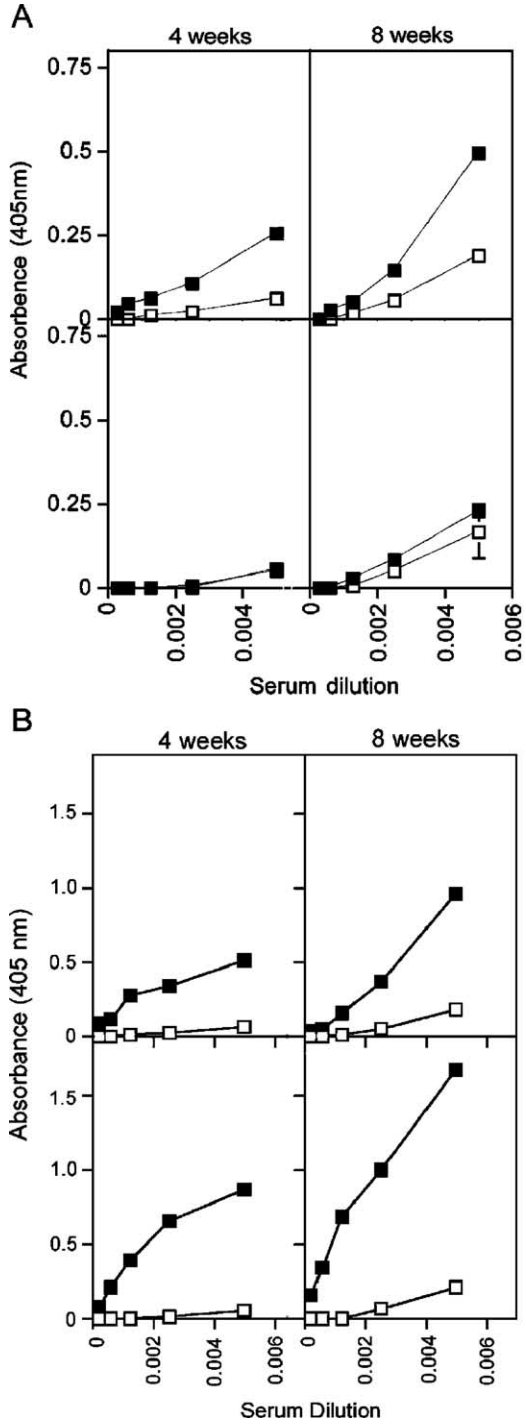

Fig. 4. The rabies virus glycoprotein-specific immune response at 4 and 8 weeks following a single administration of DNA vaccine. Groups of GKO mice (bottom panel) and C57BL/6 mice (top panel) were immunized in panel A with pCIIrab.gp and pEN.CIITA vectors (solid squares) or pEn.CIITA vector only (open squares) in panel B or with pSG5rab.gp vector (solid squares) or as a control pEn.CIITA vector (open squares). Data are shown as mean ± SD for triplicate samples. Data were analyzed using a Student's *t* test. There was a higher antibody response detected in C57BL/6 mice immunized with both pCIIrab.gp and pEN.CIITA vectors compared with mice immunized with only pEn.CIITA vector ($P \leq 0.01$). No significant difference in the immune response was observed in GKO mice immunized with pCIIrab.gp + pEN.CIITA vectors or only pEn.CIITA vector at either 4 weeks or at 8 weeks post-immunization.

nodes were isolated 72 h later. DNA and cDNA samples from individual mice were analyzed for the presence of sequences of the rabies virus glycoprotein gene by a real-time PCR. DNA in muscle and DNA and cDNA from lymph nodes could not be reliably detected by a real-time PCR in a significant number of the samples, therefore these data were not included in this study. The analysis of cDNA from the muscle samples showed some variability in the amounts detected between individual mice of a given group. Nevertheless, significantly higher ($P \leq 0.05$) copy numbers of rabies glycoprotein mRNA copies could be detected in muscle from pCIIrab.gp + pEn.CIITA co-immunized C57BL/6 mice than in the similarly vaccinated GKO mice, indicating that IFN-γ was essential to upregulate the promoter in pEn.CIITA as in its absence expression of rabies virus glycoprotein transcripts from the pCIIrab.gp vector was markedly lower (Fig. 5). The lowest amount of mRNA was detected in both C57BL/6 and GKO mice injected with pCIIrab.gp only. Samples from control animals that were not injected with a vector containing the rabies virus glycoprotein were negative in all experiments.

Responses to HIV-1 gag

To evaluate the vaccines for induction of $CD8^+$ T cell responses, we used a codon-optimized gene encoding a truncated from of the gag gene of HIV-1 for which an immunodominant $CD8^+$ T cell epitope has been identified for mice of the H-2^d haplotype. Splenocytes of BALB/c mice vaccinated with pCII.gag37 vector alone or with pCII.gag37 + pEn.CIITA vectors and mice injected with pEn.CIITA vector alone (negative control) or pCMVgag37M1-10D vector (a positive control vector in which the transgene product is driven by the CMV promoter) were tested for frequencies of gag-specific splenic $CD8^+$ T cells by intracellular cytokine staining for IFN-γ 8 to 10 weeks later. Frequencies in mice immunized with pCII.gag37 + pEn.CIITA vectors were approximately 1.5-fold higher than those obtained from mice immunized with pCII.gag37 only (Table 3). This result is in contrast to data obtained in the rabies virus system where induction of transgene product-specific antibodies by the pCII.rab.gp vector required addition of a vector expressing CIITA. There was no statistically significant difference in the magnitude of the $CD8^+$ T cell response elicited by pCMVgag37M1-10D or pCII.gag37 + pEn.CIITA vectors. In all groups, frequencies of gag-specific IFN-γ producing $CD8^+$ T cells increased upon a second immunization with the same vector or an unrelated viral vector, i.e., an E1-deleted adenovirus vector of the human serotype 5 expressing gag 37. Not surprisingly, the increase in DNA vaccine boosted mice was not as high as that observed in mice boosted with recombinant adenovirus. Upon booster immunization with either DNA or the AdHu5gag37 vector, differences in frequencies of gag-specific $CD8^+$ T cells achieved by priming with pCII.gag37 with or without pEn.CIITA became less pronounced.

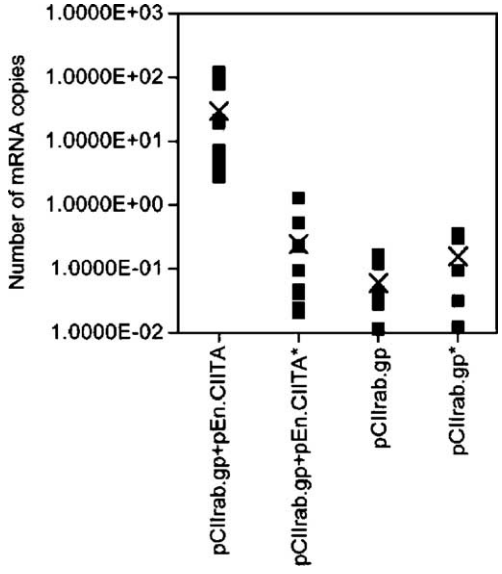

Fig. 5. Quantification of mRNA in muscle by real-time PCR using oligonucleotide primers that amplify the rabies glycoprotein gene (Reyes-Sandoval and Ertl, 2004). RNA was isolated from muscle tissue obtained from C57BL/6 mice and interferon γ knockout (GKO) (B6.129S7-Ifngt) (*) mice 72 h following IM vaccination with pCIIrab.gp vector, both pCIIrab.gp and pEn.CIITA vectors or pEn.CIITA vector only. Data shown are the number of copies of rabies glycoprotein gene per 10^6 copies of the housekeeping gene graph. Rabies glycoprotein mRNA was not detected in the mice injected with pEn.CIITA vector only. The amount of rabies glycoprotein mRNA detected in C57BL/6 mice injected with pCIIrab.gp and pEn.CIITA vectors was higher than in GKO mice injected with the same vaccines ($P \leq 0.05$). C57BL/6 mice injected with pCIIrab.gp and pEn.CIITA vectors had higher levels of rabies glycoprotein mRNA than those given only pCIIrab.gp vector ($P \leq 0.05$). There was no significant difference in the amount of mRNA detected in GKO and C57BL/6 mice immunized with pCIIrab.gp vector only. Means are indicated by X.

Table 3
Induction of HIV gag-specific IFN-γ producing $CD8^+$ T cell response in the spleen

Vaccine	$CD8^+$ T cell response following immunization (%)		
	8 weeks after prime	14 days after homologous boost with DNA vaccine	7 days after boost with 10^9 vp AdHu5gag37
pCII.gag37	0.28 ± 0.12	0.83 ± 0.03	6.3 ± 2.02
pEn.CIITA	0.01 ± 0.01	0.01 ± 0.008	0.44 ± 0.2
pCMVgag37MI-10D	0.55 ± 0.08	1.55 ± 0.5	10.39 ± 0.69
pCII.gag37 + pEn.CIITA	0.47 ± 0.05	1.06 ± 0.11	8.14 ± 3.4

Percentage of IFN-γ positive $CD8^+$ T cells in the spleen 8 weeks following a DNA vaccine prime, 14 days following homologous boost with DNA vaccine or 7 days following boost with 10^9 vp AdHu5gag37. Data are shown as the means of two separate experiments ± SD. Highest response was observed in mice immunized with pCMVgag37M1-10D ($P \leq 0.05$). However, there was no statistically significant difference in the response generated in mice immunized with pCMVgag37M1-10D or pCII.gag37 and pEN.CIITA when boosted with DNA vaccine or AdHu5gag37.

Discussion

Although DNA vaccines induce protective immunity in experimental animals, they thus far have lacked efficacy in humans. The DNA vaccine's inability to induce robust immune responses in humans may reflect recently described differences in the immune systems of mice and humans such as those of TLR expression patterns in distinct subsets of dendritic cells

described recently (Wagner, 2004). Transduction rates may differ especially upon intramuscular administration of DNA vaccines where the inoculation volume used for rodents cannot be increased proportionally to the weight and body mass of a human (that is, 0.1 ml for a 20 g mouse would translate into 300 ml for a 60 kg human). In mice, immune responses to DNA vaccination decrease upon reduction of the inoculation volume, indicating that the pressure associated with injection of a relatively large amount of fluid may facilitate transduction or alternatively may cause tissue damage that promotes activation of the innate immune system. DNA vaccines may achieve lower antigenic loads in humans due to impaired transcription. Various studies have clearly demonstrated that the use of viral promoters generally does not achieve long-term high level gene expression in vivo, even when substantial amounts of DNA are used. One of the reasons for this is promoter extinction (Qin et al., 1997). As our studies and the work of other researchers show, cytokines such as TNF-α and particularly IFN-γ, which are released as part of an innate immune response to the inoculated plasmids, play a role in the downregulation of viral promoters at both the transcriptional and posttranscriptional level in vivo (Gribaudo et al., 1993; Kerr and Stark, 1992). IFN-γ interference on a viral promoter may be more pronounced in hosts such as humans that unlike experimental mice are not maintained in a controlled laboratory environment, which is relatively free of pathogens. Consequently, these hosts may have a range of underlying infections that promote cytokine production resulting in decreased antigen production by DNA vaccines. Some eukaryotic promoters such as the MHC class II promoter are not downregulated by IFN-γ. Previous studies have shown that the MHC class II promoter is relatively weak (Xiang et al., 1997) and requires activation by CIITA (Mach et al., 1996). CIITA transcription can be upregulated by IFN-γ, LPS and IL-4 (Harton and Ting, 2000; Ting and Trowsdale, 2002). CIITA enhances MHC class II expression by coordinating the activity of a range of transcription factors including NF-Y and CREB (Zhu et al., 2000). In some cells such as B cells and immature dendritic cells, CIITA is constitutively produced, in endothelial cells, fibroblasts and presumably muscle cells, CIITA production may be induced by IFN-γ (Steimle et al., 1994). CIITA production is regulated by either three or four promoters in mice and humans respectively (Pai et al., 2002). Importantly, the IFN-γ-induced transcription of CIITA is considered to be mainly facilitated via the CIITA type IV promoter (this promoter contains a GAS element, E box and IRF 1 and 2 binding sites, all of which are associated with its IFN-γ inducibility) (Muhlethaler-Mottet et al., 1998; Muhlethaler-Mottet et al., 1997), and it has been shown that IFN-γ-induced CIITA expression precedes the expression of the MHC class II genes (Morris et al., 2002).

The results of our study clearly demonstrate that significant antibody responses to a transgene expressed by an MHC class II promoter-driven vector are generated when co-administered with a CIITA expressing vector containing either a synthetic IFN-γ-inducible promoter pEn or a viral promoter (RSV). In contrast, only a relatively weak antibody response was generated in the absence of co-inoculated CIITA. Studies in GKO mice further underscore the importance of IFN-γ in the upregulation of pEN, demonstrating that, in the absence of IFN-γ, promoter pEn activity was adversely affected as indicated by a significant decrease in the antibody response to the rabies glycoprotein transgene in these mice when co-inoculated with both pEn.CIITA + pCIIrab.gp vectors. Immune responses correlated with levels of antigen expression in muscle, which were increased in mice injected with pCIIrab.gp upon addition of the pEn.-CIITA vector. This increase appeared to be strictly dependent upon IFN-γ and was thus not observed in GKO mice that due to genetic engineering have a loss mutation for this cytokine. The correlation between levels of transcripts and by inference antigenic load produced by muscle cells may reflect that the antibody response to the DNA vaccine is at least in part driven by antigen that originates from transduced cells residing close to the site of vector administration. Results for induction of $CD8^+$ T cell responses to gag differed. Studies in BALB/c mice demonstrated that priming with both pCII.gag37+ pEn.CIITA vectors rather than just pCII.gag37 vector alone did not result in a significant increase in levels of IFN-γ expressing $CD8^+$ T cells. Furthermore, when boosted with the same DNA vaccine and particularly with a viral-vector-based vaccine (AdHu5gag37), frequencies of gag-specific $CD8^+$ T cells were high even in mice that had only been primed with the pCII.gag37 vector. This suggests that the co-administration of CIITA did not significantly affect the $CD8^+$ T cell responses in these mice. This may reflect that naive $CD8^+$ T cells are stimulated primarily by directly transduced dendritic cells in which endogenous CTIIA sponsors activation of the vector's MHC class II promoter. The plasmid driven by the native CIITA type IV promoter performed poorly as shown by the lack of strong transgene product-specific immune responses in mice injected with pCIIrab.gp + pPIV.CIITA vectors indicating that CIITA promoter IV may not be active in muscle tissue even in presence of IFN-γ. In summary, our studies show that vectors based on the MHC class II promoter induce potent immune responses (particularly B cell responses) in the presence of CIITA. Traditional DNA vaccines in which transgene product expression is driven by a strong viral promoter outperformed these novel vectors in mice at least with regard to induction of antibody responses. This may in part reflect the use of a two-vector system in which increased expression of the MHC class II-promoter driven transgene depends on co-transfection of cells with the CIITA-expressing vector. A one-vector system either encoding CIITA and the vaccine antigen under the control of the MHC class II promoter within a single vector or a vaccine vector with a promoter that is independent of a transactivator that shows enhanced activity in the presence of pro-inflammatory cytokines may result in superior immune responses. This may render such vaccines useful for larger species where pro-inflammatory cytokines induced either by the vaccine vectors or by underlying chronic infections may have a more substantial impact on the performance of vectors that are driven by viral promoters.

Materials and methods

Mice

Female 6–8-week-old C3H/He mice and BALB/c mice were obtained from Charles River (Boston, MA). IFN-γ knockout mice (GKO) strain B6.129S7-Ifngt and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were housed at the Animal Facility of The Wistar Institute.

Cell culture

Baby Hamster Kidney (BHK), NIH3T3 and L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 10% CO_2 incubator at 37 °C. HT-2 cells, an indicator cell line for IL-2 and IL-4, were grown in DMEM supplemented with 10% FBS and 10% rat concanavalin A.

Viruses

Evelyn Rokitniki Abelseth (ERA) strain of the rabies virus inactivated with β-propionolactone (ERABPL) was used at a concentration of 0.1 mg/ml. An E1/E3 deleted adenoviral vector of the human serotpye 5 expressing a truncated form of gag of HIV-1, termed AdHu5gag37, has been described previously (Fitzgerald et al., 2003).

Plasmids used in this study

Plasmids pSG5rab.gp and pCIIrab.gp have been previously described (Xiang et al., 1997). Plasmid pRep4.CIITA was a generous gift from Dr. P. van den Elsen (Leiden University, The Netherlands) (Steimle et al., 1993). The MHC class II transactivator (CIITA) (excised from plasmid pRep4.CIITA) was inserted into the pSG5.pBlue vector (this vector was constructed by the insertion of the cloning sites of pBluescript into the pSG5 vector) (Xiang et al., 1997). The IFN-γ inducible CIITA type IV promoter (PIV) was amplified from the genome of mouse cells by a polymerase chain reaction (PCR). This promoter was then cloned into the pSG5.pBlue vector upstream of the CIITA gene to create plasmid pPIV.CIITA. Plasmid pSG5LacZ has been described before. To improve efficacy of PIV while retaining its fundamental IFN-γ inducibility, the IFN-γ-responsive elements of CIITA type IV promoter, i.e., GAS, IRF-E and E box, were used to generate a hybrid promoter. The sequence of the mouse PIV containing the GAS (gamma activation site), IRF1/2 (interferon regulatory sites 1 and 2) and E box was amplified by PCR. Two copies of this PCR product were inserted in tandem upstream from a minimal TK promoter (the minimal TK promoter contained transcription-associated TATA and GC elements which are not present in the CIITA type IV promoter) to create promoter pEN. Promoter pEN and CIITA were then cloned into the pSG5.pBlue vector to create plasmid pEn.CIITA. Plasmid pCMVgag37M1-10D was most kindly provided by Dr. G. Pavlakis (National Cancer Institute, Frederick, MD) (Schneider et al., 1997). Plasmid pCII.gag37 was constructed by inserting gag37 obtained by PCR amplification from pCMVgag37M1-10D into the pCII vector downstream from the MHC class II promoter (Table 1).

In vitro assay to determine LacZ (β-galactosidase) expression

L929 cells were transfected with plasmid pSG5LacZ (LacZ inserted into the pSG5 vector). Following a 3 h incubation IFN-γ at concentrations of 100 U/ml, 50 U/ml or 1 U/ml was added. Cells were incubated for another 48 h, and lysates were obtained from them and tested in a β-galactosidase assay using standard techniques.

Immunization and bleeding of mice

Mice were inoculated by intramuscular injection of both hind legs. C3H/He mice immunized with rabies glycoprotein expressing plasmids were vaccinated with pCIIrab.gp (plasmid expressing the rabies glycoprotein driven by the MHC class II promoter) and pRep4.CIITA [plasmid expressing the MHC class II transactivator (CIITA) driven by the RSV promoter], pCIIrab.gp alone, both pCIIrab.gp and pPIV.CIITA or pCIIrab.gp and pEn.CIITA or pSG5rab.gp alone. BALB/c mice immunized with gag37 expressing plasmids were vaccinated with pCII.gag37 with and without pEn.CIITA, pEn.CIITA alone or pCMVgag37M1-10D alone. These mice were boosted using either DNA vaccine or AdHu5gag37 virus by intramuscular injection. GKO (B6.129S7-Ifngt) mice were immunized with pCIIrab.gp and pEn.CIITA or pEn.CIITA only.

Mice were bled by retro-orbital puncture, and serum was obtained for antibody determination. All experiments were repeated between 2 and 4 times. In each experiment, mice were immunized with 100 μg of each plasmid, those immunized with just one plasmid were each also vaccinated with 100 μg empty pSG5.pBlue vector (each mouse receiving a total of 200 μg DNA).

DNA vaccine preparation

Large-scale plasmid preparation was performed using the Qiagen Mega prep kit (Qiagen, Valencia, CA). Concentration of DNA was determined by spectrophotometry.

PCR and real-time quantitative PCR (RT-PCR)

Groups of five C57BL/6 and GKO (B6.129S7-Ifngt) mice were administered pCIIrab.gp, pCIIrab.gp and pEn.CIITA or pEn.CIITA only by intramuscular injection into both gastrocnemius muscles. After 72 h, mice were euthanized, and the injected muscles and draining lymph nodes (popliteal) were harvested separately for DNA and RNA extraction using TRI Reagent (Sigma, MO) following the manufacturer's protocol. Samples of mRNA were subjected to cDNA synthesis using M-MLV reverse transcriptase (Invitrogen, CA), thereafter, both DNA and cDNA samples were analyzed by real-time PCR (RT-

PCR) by previously described methods (Reyes-Sandoval and Ertl, 2004). Data were analyzed using a Student's *t* test and ANOVA.

Virus neutralization assay

Virus neutralizing antibody titers were determined using BHK-21 cells as described previously (Ertl et al., 1989). Samples were assayed in duplicate in serial threefold dilutions. Neutralization titers were determined as the reciprocal of the serum dilution causing a 50% reduction in the number of cells infected by the virus.

Enzyme-linked immunosorbent assay (ELISA) for rabies virus-specific antibodies

The ELISA was performed as previously described (Xiang et al., 1995). Briefly, microtiter plates were coated with 0.2 μg/well of ERA-BPL virus, and dilutions of sera were added. Following a 1 h incubation, plates were washed, and an alkaline-phosphatase-labeled secondary antibody was added. Finally, substrate was added, and IgG concentration was determined by measuring the optical density in an ELISA plate reader at a wavelength of 405 nm.

T helper assay

IL-2 or IL-4 released from stimulated splenocytes of vaccinated mice was detected in a biological assay (Ertl et al., 1989). Briefly, 6×10^6 splenocytes were harvested from each group of mice and grown in the presence of ERA-BPL antigen or without antigen. Cell-free supernatants were harvested 48 h later and tested for induction of proliferation of HT-2 cells (IL-2/IL-4-dependent cell line).

IFN-γ assay

Approximately 6×10^6 splenocytes were harvested from each group of vaccinated mice and cultured with or without ERA-BPL antigen. Cell-free supernatants were harvested 48 h later and tested for IFN-γ using an ELISA as previously described (Reyes-Sandoval and Ertl, 2004).

Intracellular cytokine staining

Intracellular cytokine staining was performed as described previously (Fitzgerald et al., 2003). Splenocytes were stimulated for 5 h with a peptide containing the H-2^d mouse haplotype-specific immunodominant epitope for HIV gag. Antibodies used in this assay were a FITC-labeled anti-CD8 and a PE-labeled anti-IFN-γ (BD Pharmingen, San Diego, CA).

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