Kinetics of Recombinant Adenovirus Type 5, Vaccinia Virus, Modified Vaccinia Ankara Virus, and DNA Antigen Expression In Vivo and the Induction of Memory T-Lymphocyte Responses

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While a new generation of vaccine vectors has been developed for eliciting cellular immune responses, little is known about the optimal routes for their administration or about the ramifications of the kinetics of in vivo vaccine antigen expression for immunogenicity. We evaluated the kinetics of vaccine antigen expression by real-time in vivo photon imaging and showed dramatic differences in these kinetics using different vectors and different routes of administration. Further, using a gamma interferon enzyme-linked immunospot assay to measure T-lymphocyte immune responses, we observed an association between the kinetics of vaccine antigen expression in vivo and the magnitude of vaccine-elicited memory T-lymphocyte responses. These results highlight the utility of the real-time in vivo photon-imaging technology in evaluating novel immunization strategies and suggest an association between the kinetics of vaccine antigen clearance and the magnitude of vaccine-elicited T-lymphocyte memory immune responses.

While a new generation of vaccine vectors has been created to induce cellular immune responses, we know little about how to use them to maximize the generation of memory T-lymphocyte populations. Recombinant viral vectors and plasmid DNA constructs have been shown to elicit potent cellular immune responses (2, 7, 8, 13, 15, 16, 21, 23). However, little has been done to evaluate the optimal routes of administration of these vaccine modalities and the relationship between the route of administration and in vivo vaccine antigen expression. Moreover, we do not fully understand the ramifications of the kinetics of vaccine antigen expression for immunogenicity.

The in vivo evaluation of new vaccine vectors to select optimal routes of administration, dose, and biodistribution has been difficult, requiring serial sacrifice of laboratory animals and assessment of individual organs for vaccine antigen expression. To simplify this process of vaccine evaluation, we have adopted an in vivo imaging system (IVIS) to measure the expression of luciferase by vaccine vectors. This imaging strategy harnesses the ability of the luciferase protein to catalyze the light-producing oxidation of the small molecule luciferin. Luciferin is inoculated into mice that have received luciferase-expressing immunogens, and the quantity of light emitted by this reaction is monitored in living mice (6).

The use of in vivo imaging and luciferase expression (4), a technology developed for evaluating gene therapy strategies, is well suited for studying these novel vaccine vectors. We have recently shown that IVIS can be used to monitor the distribution and kinetics of vaccine vectors in the living mouse (12). The present study was done to explore the impact of the route of vaccine administration on vaccine antigen expression. In the process of doing these studies, we observed a striking association between the kinetics of antigen expression in vivo and the induction of long-term memory T cells.

MATERIALS AND METHODS

Animals and immunizations. Six- to 8-week-old female BALB/c and athymic BALB/c nude (nu/nu) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained under pathogen-free conditions, and the experimental protocols were approved by the Harvard Institutional Animal Care and Use Committee (20). Between 10 7 and 10 9 particles of recombinant adenovirus serotype 5 (rAd5), 10 5 and 10 6 PFU recombinant vaccinia virus (rVac) (Western Reserve strain), 10 6 and 10 7 PFU recombinant modified vaccinia virus Ankara virus (rMVA), and 50 μg and 200 μg of plasmid DNA were used for immunization. Vaccines were administered in 100 μl of sterile saline solution for subcutaneous (s.c.) and intraperitoneal (i.p.) injections, in 5- to 10-μl solutions for each nostril for the intranasal (i.n.) infusions, or in two 50-μl solutions divided between the two quadriceps for the intramuscular (i.m.) injections.

Vectors. rAd5 with early region 1 and 3 (E1/E3) deleted and containing the GL2 luciferase gene (Promega, Madison, WI) was provided by Gary Nabel (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD). pGWiz-Luc was purchased from Genlantis (San Diego, CA). rMVA was provided by Mariano Esteban, Centro Nacional de Biotechnologia (Madrid, Spain). rVac and rMVA expressing luciferase were propagated and titers were determined as previously described (22).

To construct the plasmid DNA-Luc, the GL4.10 luciferase sequence (Promega) was PCR amplified and inserted into the pVRC2000 plasmid vector as described previously (3). Plasmid DNA was prepared using an endotoxin-free Qiagen Giga-prep kit (Valencia, CA).

In vivo bioluminescence measurement. Animals were injected i.p. with 100 μl of a 30-ng/ml solution of firefly luciferin (Xenogen, Alameda, CA) in phosphate-buffered saline, as well as 100 μl of a 20-ng/ml ketamine and 1.72-μg/ml xylazine mixture. After 20 min, imaging was performed using the IVIS Series 100 (Xenogen) with an integration time of 1 minute. Overlay images and luminescence measurements were made using Living Image software (version 2.50; Xenogen).

Measurement of antigen expression. To convert the in vivo relative light units (RLU) of the different vectors into the quantity of antigen expressed, we injected different amounts of recombinant luciferase protein i.m. into control mice and measured the value of the emitted light. There was a linear correlation between the amount of protein injected (10 ng to 50 μg) and the light emitted, enabling us to calculate antigen expression from the different vectors according to the following formula: concentration of antigen (in ng) = [10(log RLU − 4.21)/0.76]1/1000.
Immunological assays. Enzyme-linked immunospot (ELISPOT) assays were performed as previously described (14). A luciferase peptide pool of 67 18-mer peptides overlapping by 10 amino acids spanning the luciferase protein was synthesized by Quality Controlled Biochemicals (Hopkinton, MA).

Data analysis. The statistical significance of differences between groups was determined using the program GraphPad Prism (version 4.03). A P value of <0.05 was considered statistically significant. Correlations were identified by simple linear regression analysis.

RESULTS

The in vivo evaluation of new vaccine vectors to select optimal routes of administration, dose, and biodistribution has been difficult, requiring serial sacrifice of laboratory animals and assessment of individual organs for vaccine antigen expression. To simplify this process of vaccine evaluation, we have adopted IVIS to measure the expression of luciferase by vaccine vectors. This imaging strategy harnesses the ability of the luciferase protein to catalyze the light-producing oxidation of the small molecule luciferin. Luciferin is inoculated into mice that have received luciferase-expressing immunogens, and the quantity of light emitted by this reaction is monitored in living mice (6). We used this strategy to monitor the transgene expression of the commonly used vaccine vectors rAd5, rVac, rMVA, and DNA. These vaccine vectors were administered by four routes of inoculation using doses routinely used in mice to assess their immunogenicity (11). Having previously shown that vaccine-elicited T cells can modulate in vivo vaccine antigen expression (12), we evaluated luciferase expression profiles in T-cell-deficient athymic nude mice, in addition to wild-type mice, to document the distribution and kinetics of antigen expression in the absence of immune clearance of the vaccine vectors. Finally, each vaccine vector was evaluated at both a high dose and a low dose to ensure that the biodistribution of vaccine antigen observed in the studies reflected the true potential of the vectors.

In the rAd5-Luc-inoculated wild-type mice, high-level transgene expression was observed for approximately 1 week in the mice injected by the i.m., i.p., and s.c. routes, with considerably lower-level expression observed in those injected by the i.n. route (Fig. 1A and B). Interestingly, the T-cell-deficient athymic mice were unable to clear vaccine antigen inoculated by the i.m., s.c., and i.n. routes but were able to clear antigen inoculated i.p. (Fig. 1C and D). This finding suggests T-cell-mediated clearance of rAd5 following i.m., i.n., and s.c. administration, but not following i.p. administration.

In wild-type mice inoculated with the replication-competent rVac vector, we observed significant transgene expression for the first week following i.m. and i.p. administration, but not following i.n. and s.c. administration (Fig. 2A and B). A dramatic damping of transgene expression then occurred. There was no damping of transgene expression in the T-cell-deficient
athymic mice, and disseminated rVac replication led to the early death of the animals (Fig. 2C and D).

To complement these studies of a replication-competent poxvirus vector, we also evaluated the nonreplicating poxvirus rMVA (Fig. 3). High-level transgene antigen expression (up to 100 μg/mouse) was observed immediately following i.m., i.p., and s.c. administration of the rMVA construct, but this expression was no longer detectable by 48 h. Interestingly, these expressions were similar in wild-type (Fig. 3A) and athymic (Fig. 3B) mice. In contrast to these findings, no transgene expression was seen following i.n. administration of the rMVA construct.

In vivo transgene expression by the plasmid DNA vector was then examined (Fig. 4). Expression was highest in wild-type mice following i.m. and s.c. delivery (Fig. 4A) and persisted for 2 to 4 weeks. In contrast to these findings, high-level transgene expression never decreased in athymic nude mice inoculated with the construct by the i.m. and s.c. routes (Fig. 4B). Little measurable transgene expression was observed in wild-type or athymic mice inoculated by the i.n. and i.p. routes.

The striking difference between the clearance of transgene expression in the wild-type and athymic mice after administration of many of these recombinant vector constructs suggested
that the kinetics of this clearance might be associated with the generation of cellular immune responses. To explore this possibility, we evaluated the association between the decrease in the transgene expression after the administration of these vaccine constructs and the magnitude of the induced cellular immune response as measured by gamma interferon (IFN-γ) ELISPOT assay 3 months after the administration of the constructs. Antigen expression began to decline in wild-type mice for rAd5 at day 4, for rVac at day 7, and for DNA at day 14 (Fig. 1, 2, and 4). A statistically significant correlation was observed between these parameters (Fig. 5). This association suggests a relationship between the clearance of transgene expression and the generation of long-term memory cellular immune responses.

To evaluate the impacts of early transgene expression on long-term memory cellular immune responses for the different vectors, we examined the association of peak antigen expression in the first days after vaccine inoculation and long-term immune responses. With the exception of the i.m. route of the DNA vector, we found no association between expression during the first 2 days following immunization and long-term immune responses (Fig. 6).

**DISCUSSION**

To test if the magnitudes of vaccine-elicited immune responses are associated with the kinetics of vaccine antigen expression in vivo, we evaluated the kinetics of transgene expression with plasmid DNA, rAd5, rVac, and rMVA vectors delivered via i.m., i.p., s.c., and i.n. routes. We observed different in vivo patterns of luciferase transgene expression by different vectors. Surprisingly, we did not observe a strict relationship between the peak vector antigen expression and immunogenicity. Indeed, peak antigen expression levels were roughly comparable between DNA, rAd5, and poxvirus vectors, while rAd5 produced significantly higher-frequency cellular immune responses. Additionally, we observed that striking differences in expression were also found in animals inoculated with the same vector via different routes. These studies, therefore, illustrate the importance of vector choice and route of delivery for antigen expression in vivo.

These studies also highlight the utility of the IVIS technology for optimizing vaccine delivery. This technology provides a rapid, simple approach for selecting the optimal route of delivery for a particular vaccine platform. Its quantitative precision allows the careful evaluation of the contribution of the kinetics of vaccine antigen expression and immunogenicity.

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**FIG. 4.** In vivo imaging of luciferase expression following inoculation with 50 μg plasmid DNA-Luc in wild-type and nu/nu mice. The mice (n = 4) were inoculated by i.m., i.p., s.c., and i.n. routes. RLU were measured in wild-type (A) and nu/nu (B) mice and transformed into protein expression using recombinant luciferase protein as a standard. The data are expressed as mean values (± standard errors of the mean). The time after DNA inoculation is shown in a log scale to highlight early events.

**FIG. 5.** Association between damping of late (>3 days) vaccine antigen expression and vaccine-elicited T-cell responses. (A and B) Luciferase-expressing rAd5 (A) and rVac (B) were inoculated at doses of 10⁸ and 10¹⁰ virus particles or 10⁵ and 10⁶ PFU, respectively, by the i.m., i.p., i.n., or s.c. route (four mice/dose/route). Luciferase expression was then measured between day 4 and day 56. (C) Mice (n = 16) were also inoculated with 50 μg DNA-Luc by the i.m. route. Luciferase expression was measured between 2 and 4 weeks following vaccination. T-cell immune responses were measured 3 months after vaccination by IFN-γ ELISPOT assay following exposure of splenocytes to a pool of luciferase peptides. SFC, spot-forming cells.
The studies performed in athymic mice underscore the central role of T lymphocytes in modulating the in vivo expression of vaccine antigen. It is interesting, however, that this role differs with different vectors, as well as with different routes of administration. Thus, T lymphocytes play a central role in damping rAd5 transgene expression when the vaccine is delivered i.m. and s.c. but play a less significant role when the same recombinant vector is administered i.p. (Fig. 1). In the absence of functional T lymphocytes, the rVac constructs caused the death of the inoculated animals, demonstrating a critical role for T-cell immunity in this vector system (Fig. 2). Surprisingly, the clearance of vaccine antigen from the rMVA-inoculated mice was not affected by the absence of T lymphocytes (Fig. 3).

For all vector modalities tested, the kinetics of transgene clearance were closely associated with the long-term immune responses elicited. As memory T-cell responses are derived from the same cell populations as primary T-cell responses (24), there should be a strong association between primary and memory immune responses. Interestingly, however, it was the rate of clearance of transgene expression, rather than the kinetics of its initial peak expression, that was most strongly associated with long-term memory T-cell responses of any route for this vector (Fig. 3). By contrast, no detectable long-term immune responses were observed by ELISPOT assay for routes of immunization in which antigen expression levels were the same in both wild-type mice and athymic mice.

We also evaluated associations between very early antigen expression and long-term immune responses. No association was observed for most of the evaluated vectors. Surprisingly, however, our data indicate that early high levels of antigen expression might be important in increasing the plasmid DNA vaccine-induced long-term memory immune response for the i.m. route of vaccination (Fig. 6). There are two mechanisms that could explain this phenomenon, either alone or jointly. First, since direct major histocompatibility complex (MHC) class II presentation of DNA antigen through myocytes to T cells is normally not very efficient because of the relatively low level of expression of the MHC class II molecules on myocytes (1, 10, 17, 19), high antigen expression might overcome this deficiency. Secondly, high expression of antigen might increase inflammatory signals delivered from the innate immune system, which was recently shown to be necessary to increase MHC class II protein expression (1, 19, 25). Both mechanisms might increase the direct presentation of antigen to T cells by myocytes, which appears to be the dominant trigger for the eventual damping of antigen expression. Indeed for DNA vaccination, direct antigen presentation to T cells might be more important than the more traditional antigen presentation by antigen uptake of professional antigen-presenting cells (9, 18, 25) or antigen presentation of myocytes to antigen-presenting cells (5).

Most importantly, our data suggest that vaccine antigen clearance is mostly dependent on the magnitudes of adaptive immune responses and that these responses are associated with the magnitudes of the elicited memory cellular responses.

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