Lack of an Immune Response against the Tetracycline-Dependent Transactivator Correlates with Long-Term Doxycycline-Regulated Transgene Expression in Nonhuman Primates after Intramuscular Injection of Recombinant Adeno-Associated Virus

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We previously documented persistent regulation of erythropoietin (Epo) secretion in mice after a single intramuscular (i.m.) injection of a recombinant adeno-associated virus (rAAV) vector harboring both the tetracycline-dependent transactivator (rtTA) and the Epo cDNA (D. Bohl, A. Salvetti, P. Moullier, and J. M. Heard, Blood 92:1512-1517, 1998). Using the same vector harboring the cynomolgus macaque Epo cDNA instead, the present study evaluated the ability of the tetracycline-regulatable (tetR) system to establish long-term transgene regulation in nonhuman primates. The vector was administered i.m., after which 5-day induction pulses were performed monthly for up to 13 months by using doxycycline (DOX), a tetracycline analog. We show that initial inductions were successful in all individuals and that there was a tight regulation and a rapid deinduction pattern upon DOX withdrawal. For one macaque, regulation of Epo secretion was maintained during the entire experimental period; for the five remaining macaques, secreted Epo became indistinguishable from endogenous Epo upon repeated DOX inductions. We investigated the mechanism involved and showed that, except in the animal in which secretion persisted, delayed humoral and cellular immune responses were directed against the rtTA transactivator protein associated with the reduction of vector DNA in transduced muscles. This study provides some evidence that, when the immune system is not mobilized against the rtTA transactivator, the tetR-regulatable system is able to support long-term transgene regulation in the context of an rAAV in nonhuman primates. In addition, our results suggest potential improvements for vector design.

Recombinant adeno-associated virus (rAAV) vector-mediated gene transfer in skeletal muscle of mice (36), dogs (13), nonhuman primates (8, 37), and hemophilia patients (16) is well tolerated and is associated with long-term expression. As such, it becomes possible to evaluate strategies which allow long-term transgene regulation; such strategies are likely to be required for therapeutic applications and in some instances for safety reasons. A rather limited number of clinically translatable regulatory systems are available. They all have in common the use of chimeric transactivators, the activity of which is controlled by drugs including tetracycline (11), mifepristone (35), ecysdsone (23), and rapamycin (25).

The rapamycin-regulatable system uses rapamycin or its analog to bring together the functional unit of bipartite chimeric transcription factor ZFHD1/FKBP-FRAP/Pd65 (25). Their corresponding cDNAs have been included in an rAAV vector and injected intramuscularly (i.m.) in macaques along with a second rAAV harboring the erythropoietin (Epo) cDNA under the control of a ZFHD1-dependent promoter. This resulted in long-term regulation of Epo secretion in mice and regulation for up to 3 months in one rhesus macaque out of three (37).

The repressor of the Tn10 tetracycline resistance operon of Escherichia coli (tetR) recognizes its operator (tetO) with high specificity (14). The interaction between repressor and operator is efficiently prevented by tetracycline and especially by doxycycline (DOX), which binds to tetR with high affinity. A tetR mutant exhibits a reverse phenotype requiring DOX for binding to the tetO operator (12). By fusing the latter with the C-terminal portion of VP16 of herpes simplex virus, the resulting transactivator, rTA, which efficiently trans-activates Ptet, the minimal promoter fused downstream of an array of tetO sequences (11), was obtained. The presence of DOX activates transcription.

We and others have found persistent Epo regulation in mice after a single i.m. injection of an rAAV vector harboring the tetracycline-dependent transactivator (rtTA) (3) or the tetracycline-repressed transactivator (tTA) (24) and the Epo cDNA.

The present study was designed to evaluate, in nonhuman primates, the ability for the tetracycline regulatable (tetR) system to establish long-term transgene regulation in the context of an rAAV vector. Six macaques received i.m. the rAAV vector, harboring simultaneously the rtTA and the homologous
Epo cDNA cassettes. This study documents the levels of Epo secretion following DOX, a tetracycline analog, administration for up to 13 months.

MATERIALS AND METHODS

rAAV production and administration. Production of both the construct (Fig. 1) and vector was recently described (8). The number of vector genomes per milliliter was determined by dot blot hybridization as described previously (27). Animal care and i.m. delivery of rAAV were conducted as described previously (8). Six animals, corresponding to Mac 3 through Mac 8 (described in reference 8) (Table 1), were studied.

Epo induction and monitoring. Because we found that water intake varied substantially among the animals, DOX (Vibraoneuse; Pfizer), a tetracycline analog, was given intravenously (i.v.; 10 mg/kg of body weight). The induction protocol started 2 months after vector administration and consisted of a 5-day induction pulse repeated essentially once every month. Serum cynomologus monkey interleukin-2 (IL-2) and vector genomes per g of muscle were measured by enzyme-linked immunosorbent assay (ELISA; Quantitative IVD kit; R&D Systems).

DNA analysis. Southern blot analysis, 5 or 10 ng of high-molecular-weight DNA extracted from muscle biopsy samples was digested with BanHI (Fig. 1) and further processed as described previously (3). Blots were hybridized with [32P]dCTP random-primed labeled Epo, rtTA, or tetO-CMV (tetracycline-responsive) vector (not represented). ITR, BamHI restriction sites and resulting fragments are indicated. The transcriptional SV40 polyadenylation signal; CAG, CAG promoter (22).

TABLE 1. Characteristic of the rAAV doses injected and Epo regulation outputs

<table>
<thead>
<tr>
<th>Animala</th>
<th>rAAV</th>
<th>Doseb (ip/kg)</th>
<th>Peak value of Epo (mIU/ml)</th>
<th>No. of rEpoc peaks</th>
<th>Detection of anti-rEpo antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac 3</td>
<td>cmET (LTR)</td>
<td>2.5 × 1011</td>
<td>ND</td>
<td>ND</td>
<td></td>
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<tr>
<td>Mac 4</td>
<td>cmET (LTR)</td>
<td>8.0 × 1011</td>
<td>12</td>
<td>4</td>
<td>+</td>
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<tr>
<td>Mac 5</td>
<td>cmET (CAG)</td>
<td>3.0 × 1010</td>
<td>13</td>
<td>2</td>
<td>+</td>
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<tr>
<td>Mac 6</td>
<td>cmET (CAG)</td>
<td>1.0 × 1010</td>
<td>65</td>
<td>2</td>
<td>+</td>
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<tr>
<td>Mac 7</td>
<td>cmET (CAG)</td>
<td>4.0 × 1010</td>
<td>13</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>Mac 8</td>
<td>cmET (CAG)</td>
<td>4.0 × 1010</td>
<td>17</td>
<td>Persisting</td>
<td>ND</td>
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</table>

a See reference 8.
b ip., infectious particles.
c First induction.
d Epo, recombinant Epo.

Epo titers were determined by sandwich ELISA using polyclonal antibodies against Epo and rEpo as previously described (8).

Peptide synthesis. rAAV was produced by incubating control adenovirus Ad-LacZ with DOX (Vibraoneuse; Pfizer), a tetracycline analog, was given intravenously (i.v.; 10 mg/kg of body weight). The induction protocol started 2 months after vector administration and consisted of a 5-day induction pulse repeated essentially once every month. Serum cynomologus monkey interleukin-2 (IL-2) and vector genomes per g of muscle were measured by enzyme-linked immunosorbent assay (ELISA; Quantitative IVD kit; R&D Systems).

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CD68 (PG-M1 clone; Valbiotech); cryosections were subsequently incubated with anti-mouse antibodies by using a horseradish peroxidase- or AP-based EnVision kit (Dako).

**Statistical analysis.** Statistical analysis of the IFN-α/H9253 ELISPOT counts was performed by using the Student t test. A P value of less than 0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

The introduction of the complete tetR system in one single rAAV vector (Fig. 1) (8) provides a unique opportunity to evaluate its full potential in vivo in the context of the AAV inverted terminal repeats (ITRs). The initial DOX pulse displayed an important and consistent feature: as soon as 48 h after DOX initiation, Epo secretion reached a plateau, returning to baseline level in less than 5 days upon DOX withdrawal (Fig. 3). A delayed reticulocyte burst correlated remarkably well with the Epo secretion pattern, confirming the tight regulation achieved (Fig. 2c and d and 3). By comparison, the rapamycin-regulatable system harbored in adenovirus or rAAV vectors displayed a prolonged deinduction (10 to 15 days) of the system to basal expression (26, 37). Possible explanations could be a slower degradation of the transgene mRNA molecules or the presence of intracellular rapamycin (1). A similar slow deinduction pattern is obtained by using the progesterone-regulatable system, for which the inducer RU486 exhibits a long half-life and a poor diffusion within tissues (30). Although, for the tetR-regulatable system, the tight degree of regulation was previously noted in vitro (1), our data provide evidence that this feature can be translated in nonhuman primates. Of note, unlike other activators, such as steroids or rapamycin, that have a broad spectrum of activity in mammals, tetracycline's high affinity for tetR enables the use of this antibiotic at concentrations that cause little adverse effect. The recently described rTA variant M2 is fully induced in vitro at about a 10-fold-lower concentration of DOX than the one presently used (33). Therefore, it may represent a more attractive alternative in the future.

We next investigated whether the tetR system supports long-term Epo regulation in nonhuman primates. To allow accurate assessment and reproducible DOX intake, animals were subjected monthly to a 5-day pulse of DOX i.v. injections. Since they were occasionally bled to avoid high hematocrit discomfort (Fig. 2c and d), the main outputs considered were serum Epo concentration (Fig. 2a and b) and reticulocyte counts (Fig. 2c and d and Table 1). The data show that induction of Epo secretion and the subsequent reticulocyte burst upon DOX administration vanished with time and were lost after two to five pulses depending on the individual (i.e., 4 to 7 months)

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**FIG. 2.** Epo (a and b) and hematocrit (dotted lines) and reticulocyte (solid lines) (c and d) levels in representative animals Mac 6 and Mac 8. Solid arrows (a and b), DOX administration. Animals were occasionally bled (open arrows [c and d]). Ab (rTA), detection of anti-rTA antibodies in successive serum samples; c+, positive control consisting in a commercial specific MAb against rTA; c−, negative control obtained from sera prior to rAAV administration.

**FIG. 3.** Epo and reticulocyte levels, obtained from the last induction peak from Mac 8 (Fig. 2a) and determined on a daily basis upon DOX i.v. administration for 5 days (dotted area) and subsequent withdrawal.
after rAAV injection). However, one animal (Mac 8) displayed a remarkably sustained DOX-regulated Epo secretion and hematocrit elevation during the entire 13-month experimental period corresponding to 11 successive inductions (Fig. 2a and c). In previous studies with mice, long-term DOX-mediated regulation was achieved in the context of an AAV vector administered i.v. using either the rapamycin-regulatable system (26) or a DOX-regulated expression cassette (3, 24). Importantly, in none of these studies could cellular or humoral immune responses against the transactivator protein be detected. However, this possibility was investigated with our primates since the rtTA transactivator was the sole AAV-encoded component of the rtTA-directed immune response by IFN-γ ELISPOT assays. LNMCs from inguinal draining lymph nodes in the presence of autologous DCs expressing the rtTA antigen (LNMC/DC-rtTA [a and c]) or GFP (LNMC/DC-GFP [b]). Herpesvirus papio-transformed B lymphoblastoid cell lines (papio B) were derived from PBMCs of Mac 6 and Mac 8 and were then infected with either vaccinia virus vtetR (papio B + vtetR) or control wild-type vaccinia virus (papio B + vWR). Spot quantification is described in Materials and Methods. Eff, effector cells.

FIG. 4. Muscle biopsy sample obtained from Mac 6 6 months after rAAV injection. Shown is a phenotypic characterization of the cellular infiltrates. (a) Hematoxylin and eosin staining. Magnification, ×100. (b) Anti-CD68-AP. Magnification, ×200. (c) Anti-CD8-horseradish peroxidase. Magnification, ×300. (d) Anti-CD4-AP. Magnification, ×400.

FIG. 5. Cytokine production can be measured at the single-cell level by using the ELISPOT technique, allowing calculation of T-cell frequencies. Shown is an IFN-γ ELISPOT assay using autologous LNMCs from Mac 6 and Mac 8, obtained from inguinal draining lymph nodes in the presence of autologous DCs expressing the rtTA antigen (LNMC/DC-rtTA [a and c]) or GFP (LNMC/DC-GFP [b]). Herpesvirus papio-transformed B lymphoblastoid cell lines (papio B) were derived from PBMCs of Mac 6 and Mac 8 and were then infected with either vaccinia virus vtetR (papio B + vtetR) or control wild-type vaccinia virus (papio B + vWR). Spot quantification is described in Materials and Methods. Eff, effector cells.

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> 0.1) (Fig. 5). In contrast, vtetR-transduced autologous B- papio from Mac 8 was unable to stimulate IFN-γ production from DCrtTA-stimulated LNMCs (46 ± 6, 11 ± 8, and 5 ± 2 IFN-γ spots per 5 × 10^4, 1 × 10^4, and 5 × 10^3 cells, respectively) compared to the control vWR-transduced autologous B-papio (34 ± 6, 6 ± 3, and 1 IFN-γ spots) (P > 0.1) (Fig. 5). Identical patterns were obtained by using rtTA or control GFP-expressing immortalized autologous fibroblasts from Mac 8 as stimulating cells for IFN-γ production (41 ± 16 and 53 ± 1 IFN-γ spots/5 × 10^4 cells, respectively [P > 0.1]).

The results obtained with Mac 6 were reproduced 1 month later (i.e., 17 months after vector injection) in a separate set of experiments using various concentrations of autologous B-papio and freshly extracted LNMCs (not shown). Altogether, the data suggested that at least in Mac 6 specific humoral and cellular immune responses correlated with the disappearance of regulated Epo secretion. Conversely, the lack of a detectable immune reaction against the rtTA in Mac 8 was associated with the sustained transgene regulation. A similar progressive extinction of rapamycin-regulated Epo secretion was found in rhesus monkeys by Ye et al., but, although they suspected that the highly antigenic protein domains that were incorporated into the NH2 termini of both transcription factors could be the targets for a destructive immune response, no evidence for this was provided at that time (37).

Although we cannot formally rule out the possibility that partial silencing of Epo and rtTA expression occurs in vivo in macaques, in relation to the actual molecular status of the rAAV vector (21), the emerging scenario accounting for the progressive loss of Epo secretion upon DOX administration is the rise of an rtTA-directed immune response responsible for a cell-mediated destruction of the genetically modified myofibers. To support this hypothesis, Southern blot analyses were performed on total DNA extracted from muscle biopsy samples obtained from Mac 3, Mac 4, Mac 7, and Mac 8 15 to 18 months p.i. Samples were also collected from Mac 5 (6 and 12 months p.i.) and Mac 6 (1, 6, and 12 months p.i.). Muscle DNA was digested with BamHI, which releases the Epo, bidirectional SV40 polyadenylation signal, and rtTA sequences (0.6, 0.6, and 1 kb, respectively; Fig. 1). An rtTA probe (Fig. 6a) was used to show that a band of 1 kb was present in Mac 6 1 month p.i. (lane 1), but the band could no longer be detected at 6 and 12 months p.i. (lanes 2 and 3). Similarly, no signal could be found in Mac 3 and Mac 4 18 months p.i. (not shown). A 1.4-kb band, corresponding to the apparent sizes of single-stranded rAAV vector genomes (see control lane corresponding to ~10^6 vector genomes, Fig. 6b, lane 7) was consistently detected by using either rtTA (lane 1) or tetO-CMV (not shown) probes 1 month p.i. in Mac 6 samples but not in subsequent DNA samples. The same observation was also made for Mac 5. Most importantly, a DNA sample collected from Mac 8 15 months p.i. showed the persistence of 0.2 to 0.3 vector copy per equivalent haploid cellular genome (Fig. 6a, lane 4). Furthermore, in the same animal and using a tetO-CMV probe (Fig. 6b), a band of ~1.8 kb was detected, compatible with a head-to-tail concatamer with approximately one ITR deleted (lane 6). The same structure could also be seen as a faint signal for Mac 7 15 months p.i., equivalent to ~0.05 vector copy per equivalent haploid cellular genome (lane 5). Despite the lack of intermediate biopsy samples for all individuals, the molecular status of vector DNA detected at early and late time points was in agreement with previous reports for mice, in which the input single-stranded DNA is either converted to double-stranded forms (9, 34) or recruited to form duplex structures made of plus and minus single-stranded genomes (20) or both. The detection of head-to-tail junctions was also in agreement with previous reports for mice and dogs (10, 13). Whether the persisting concatemerized vector DNA found in Mac 8 is episomal and/or integrated is another issue which remains to be determined.

Altogether, our data show that rAAV administered in Mac 8 (i) failed to elicit a detectable immune reaction against the rtTA transactivator protein, (ii) persisted as head-to-tail concatemers for at least 16 months, and (iii) was associated with stable regulated Epo expression. Conversely, we found that a humoral response against the rtTA in Mac 4 through Mac 7 and vector DNA in muscle biopsy samples were undetectable 15 months p.i. (except for a faint signal in Mac 7) and that all of these animals displayed a transient regulated Epo expression. Mac 6, in which we were able to document humoral and cellular immune responses against the rtTA, exhibited at the same time a quantitative reduction of the rAAV copy number at the injection sites. The initial detection of vector DNA 1 month p.i. and its absence 6 and 12 months p.i. (Fig. 6, lanes 1 to 3) correlated with the decline of DOX-regulated Epo secretion, suggesting that one possible mechanism involved was a progressive immune-mediated destruction of the genetically modified myofibers. Although proteins secreted from an ectopic site may have biochemical features different from those of their counterpart produced at the physiological site (2), we...
found no evidence for an immune response directed against cynomolgus macaque Epo and none of the primates developed anemia as a result of cross-neutralization, unlike findings described previously (32). As suspected for the rapamycin-regulatable system, the heterologous nature of the rtTA makes it a potent candidate for triggering the host immune system, at least in primates but not in the murine model (26, 37). The present study based on (i) our PCR (8) and RT-PCR data (Fig. 7) showing detectable transgenes and transcripts in rAAV-injected muscles from all animals tested at a time when Epo induction is undetectable, (ii) the delayed onset of the immune reaction to the rtTA, and (iii) the mild mononuclear infiltrates associated with a relatively weak IFN-γ secretion, detectable only when using in vitro-activated LNMCs and not PBMCs, suggests that the immune reaction in this setting takes place at a rather slow pace and is of moderate magnitude. Thus, the complete immune-mediated removal of the transduced cells may not be achieved, at least by 15 months p.i., but is sufficient so that the number of transduced cells fall off to a critical threshold where regulated Epo expression becomes indistinguishable from endogenous Epo expression. This scenario would reconcile our sensitive RT-PCR and PCR (8) analyses and our Southern blotting data. Furthermore it was proposed that the decline of AAV-transduced gene expression in vivo would occur in a chronic way due to the limited activation of T-cell immunity (38). Whether expression in Mac 8 would follow the same model but at an even slower pace or whether a tolerant status toward the rtTA was achieved in this animal remains unknown. The fact that Mac 7 and Mac 8 developed neutralizing antibodies against the rAAV capsid (not shown) suggests that they were both immunocompetent for other nonself peptides. Nonetheless, they exhibited different immunological outcomes although they received identical doses or volumes of the same vector stock. Such a discrepancy could be related to individual genetic factors, such as major histocompatibility complex haplotype affinity for rtTA epitopes in the outbred animal. This discrepancy, if valid, underscores the importance of preclinical studies with nonhuman primates as opposed to inbred murine models, where an elaborate immune response against the rtTA was searched for and never found (3, 24).

Previous studies described rAAV vectors as nonimmunogenic, allowing long-term transgene expression in vivo (for a review, see reference 31). The basis for escape from immune recognition of nonself antigens expressed from rAAV relies, in part, on the observation that these vectors are relatively inefficient at transducing mature DCs in vitro (15) and in vivo (28). However, several conflicting reports suggested that the rAAV inability to transduce mature DCs does not necessarily result in stable gene transfer; these reports delineate additional factors implicated in the induction of cytotoxic T lymphocyte responses by rAAV vectors. Among them, the route of administration (4), the transgene involved (4, 19), the physiological status of the target tissue at the time of the rAAV injection (5), and whether transgene transcription is ubiquitous or restricted (5) appear as potent factors with respect to the host immune response after rAAV-mediated gene transfer. Recently, extracellular shedding of the transgene product and subsequent uptake and cross-presentation by local APC were documented as constituting an additional mechanism for rAAV-mediated antigen-specific T-cell activation and target cell destruction (28, 29). Whether, in our macaques, the priming of the immune response against rtTA was initiated by direct transduction of APC such as DCs or by cross-priming after recapture of soluble rtTA antigens or both is difficult to ascertain. The fact that rAAV vector DNA was found by PCR for several months in CD2+ and CD14+ PBMCs and lymph nodes in all animals (8) provides a possible experimental link with a recently proposed scenario (5, 38) in which an rAAV vector might be able to initiate a cellular response to the transgene product if enough AAV-transduced monocyte or DC precursors (18) are recruited. Additionally, this scenario proposes that the T-cell-mediated immune response to the neoantigen is probably induced by a threshold of AAV vector-transduced immature DCs. Translated to our study, the AAV vector would concentrate in the draining lymph nodes after i.m. injection (8), with subsequent transduction of monocyte or DC precursors and/or cross-priming of other APC. Factors such as vector stock impurities and i.m. injury secondary to vector injection or muscle biopsy could represent effective stimuli for activation of T-cell immunity in a favorable major histocompatibility complex haplotype, although activation would possibly be modulated by the slow rise in gene expression in the AAV context (31).

While understanding the mechanism of the AAV vector-mediated immune response in nonhuman primates remains necessary, our current strategy to circumvent the immune system relies on the tissue-restricted expression of the rtTA. Results for Mac 8 provided the “gold standard” by establishing that a clinically compatible regulatable system can be functional in the context of a single AAV vector in nonhuman primates for more than a year.

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