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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

David Favre,[1] Véronique Blouin,[1] Nathalie Provost,[1] Radec Spisek,[2] Françoise Porrot,[3] Delphine Bohl,[4] Frederic Marmé,[5] Yan Chérel,[6] Anna Salvetti,[1] Bruno Hurtrel,[3] Jean-Michel Heard,[4] Yves Rivière,[3] and Philippe Moullier[1]*

INSERM ERM 0105[1], INSERM U419,[2] and Laboratoire d'Anatomie Pathologie, INRA UR 703, Ecole Nationale Vétérinaire,[6] Nantes, and CNRS URA1930[3] and Laboratoire Rétrovirus et Transfert Génétique,[4] Institut Pasteur, Paris, France, and ZMBH, Universitaet Heidelberg, D-69120 Heidelberg, Germany[5]

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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), ecdysone (23), and rapamycin (25).

The rapamycin-regulatable system uses rapamycin or its analog to bring together the functional units of bipartite chimeric transcription factor ZFHD1/FKBP-FRAP/p65 (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 Tn*10* 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, rtTA, which efficiently *trans*-activates P_{tet}, 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

* Corresponding author. Mailing address: Laboratoire de Thérapie Génique, INSERM ERM 0105, Bâtiment Jean Monnet, CHU Hôtel-Dieu, 44035 Nantes Cedex 01, France. Phone: (33) 2 40 08 74 90. Fax: (33) 2 40 08 74 91. E-mail: moullier@sante.univ-nantes.fr.

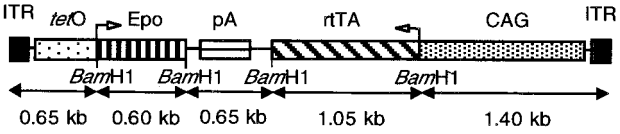

FIG. 1. Structure of the AAVcm-ET(CAG) vector. pA, bidirectional SV40 polyadenylation signal; CAG, CAG promoter (22). *Bam*HI restriction sites and resulting fragments are indicated. The CAG promoter was replaced by the retroviral MFG long terminal repeat (7) in the AAVcm-ET(LTR) vector (not represented). ITR, inverted terminal repeat; Epo, erythropoietin cDNA from *Macaca fascicularis*.

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 (Vibraveineuse; 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 macaque Epo cDNA was measured by enzyme-linked immunosorbent assay (ELISA; Quantikine IVD kit; R&D Systems).

DNA analysis. For Southern blot analysis, 5 or 10 μg of high-molecular-weight DNA extracted from muscle biopsy samples was digested with *Bam*HI (Fig. 1) and further processed as described previously (3). Blots were hybridized with [^{32}P]dCTP random-primed labeled Epo, rtTA, or tetO-CMV (tetracycline-responsive element reiterated seven times regulating the minimal cytomegalovirus immediate-early promoter) probes (Readiprime II; Amersham).

RNA analysis. Liver and muscle samples (40 mg) were treated with an RNA extraction RNAeasy minikit (Qiagen) according to the manufacturer's instructions. Total RNA was denatured for 10 min at 70°C and then chilled on ice. First-strand synthesis and subsequent PCR amplification from 1 μg of total RNA were performed by standard procedures. PCR primers were designed to amplify a 577-bp fragment (upstream primer, 5′-CCACGCTGTTTTGACCTCCATAG-3′; downstream primer, 5′-GTGTCAGCAGTGATGGTTCGGAG), a 413-bp fragment (upstream primer, 5′-CAGAGCCAGCCTTCTTATTCG; downstream primer, 5′-ACTCGAAGTCGGCCATATCC), and a 250-bp fragment (upstream primer, 5′-CACCAGCAAGCTTGCGACC; downstream primer, 5′-TGCTGG ATTACATCAAAGCAC) for Epo, rtTA, and hypoxanthine ribosyltransferase (HPRT), respectively. After 5 min at 94°C, each sample was subjected to the following amplification cycle: 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C for 40 cycles and then 10 min at 72°C. Negative controls for the reverse transcription (RT) reaction consisted in either omitting reverse transcriptase in the reaction mixture or an RT reaction on mock-transduced muscles. Control for RNA extraction was provided by HPRT RT-PCR.

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

Animal[a]	rAAV	Dose[b] (i.p./kg)	Peak value of Epo[c] (mIU/ml)	No. of rEpo[d] peaks	Detection of anti-rtTA antibodies
Mac 3	cmET (LTR)	2.5×10^9	ND[e]		ND
Mac 4	cmET (LTR)	8.0×10^9	12	4	+
Mac 5	cmET (CAG)	3.0×10^9	13	2	+
Mac 6	cmET (CAG)	1.0×10^{10}	65	2	+
Mac 7	cmET (CAG)	4.0×10^9	13	5	+
Mac 8	cmET (CAG)	4.0×10^9	17	Persisting	ND

[a] See reference 8.
[b] i.p., infectious particles.
[c] First induction.
[d] rEpo, recombinant Epo.
[e] ND, not detectable.

Humoral immune response. Detection of anti-rtTA antibodies was conducted as follows. Two hundred nanograms of purified tTA2(synth.)-His_6 protein was subjected to sodium dodecyl sulfate–14% polyacrylamide gel electrophoresis and then transferred to a Hybond ECL nitrocellulose membrane (Amersham). Membranes were then saturated and subsequently incubated for 2 h at room temperature either with a tetR mouse monoclonal antibody (1:1,000; Clontech) used as a positive control or with the experimental macaque sera (1:100). Detection used a peroxidase-conjugated goat anti-mouse antibody (1:2,000; Dako) or a goat anti-rhesus monkey immunoglobulin G (Southern Biotechnology Associates, Birmingham, Ala.) followed by enhanced chemiluminescence detection.

Cellular immune response. (i) Vectors and cells. Recombinant vaccinia virus (vtetR) and control wild-type vaccinia virus (vWR) were obtained from P. Traktamn (Milwaukee, Wis). vtetR encodes the DNA binding domain of rtTA. Recombinant adenoviruses Ad-rtTA (kindly provided by K. Walsh, Boston, Mass.), Ad-GFP, and Ad-LacZ encode rtTA, the green fluorescent protein (GFP), and β-galactosidase, respectively. Herpesvirus papio-transformed B lymphoblastoid cell lines (papio-B) were derived from peripheral blood mononuclear cells (PBMCs) of Mac 3 through Mac 8 obtained as described previously (8). Immortalized primary fibroblasts were obtained after a cutaneous biopsy on each macaque (except for Mac 6) by using a recombinant Moloney murine leukemia virus encoding the large T antigen of simian virus 40 (SV40) (17). PBMCs and lymph node mononuclear cells (LNMCs) were isolated as described previously (8). Dendritic cells (DCs) were derived from purified $CD14^+$ cells by using antibody-coated microbeads and magnetic separation (Milteny Biotec). Selected $CD14^+$ cells were cultured for 6 days in the presence of 1,000 U of granulocyte-macrophage colony-stimulating factor (Leucomax; Novartis)/ml and 50 ng of interleukin-4 (IL-4) (Biosource)/ml in RPMI medium with L-glutamine (Gibco-BRL) and 1% autologous plasma. On day 6, DCs were transduced with either Ad-rtTA (DC.rtTA) or control Ad-GFP (DC.GFP) or Ad-LacZ (DC.LacZ). DC maturation was then obtained in the presence of 5 ng of tumor necrosis factor alpha (R&D Systems)/ml and 25 μg of poly(I:C) (Sigma)/ml for 24 h. Monitoring GFP expression indicated that >90% of the mature DCs were routinely transduced. Mature DCs were counted, and their viability was assessed either by trypan blue exclusion or by propidium iodide staining. Phenotypic analysis was done by fluorescence-activated cell sorter with monoclonal antibodies (MAbs) directed against CD14, CD83, HLA-DR (Immunotech), CD80, and CD86 (Pharmingen) antigens. In vitro stimulation of PBMCs and LNMCs was carried out at a ratio of PBMCs or LNMCs to genetically modified mature DCs of 10 to 1 in 48-well plates for 24 h in RPMI medium–10 ng of IL-7/ml–10 pg of IL-12 (R&D Systems)/ml–5% autologous plasma. Cells were then maintained for 10 days in RPMI medium–20 IU of IL-2 (Chiron)/ml–10% autologous plasma.

(ii) IFN-γ ELISPOT An enzyme-linked immunosorbent spot assay (ELISPOT) as adapted from reference 6 was used to detect rtTA-specific gamma interferon (IFN-γ)-producing T cells from DC.rtTA-stimulated LNMCs. Briefly, nitrocellulose-coated 96-well plates (MAHA N45; Millipore) were coated overnight at 4°C with 50 μl of MAb MD-1 (anti-IFN-γ MAb; BioSource) at 4 μg/ml. After being washed and saturated in 5% fetal calf serum, genetically modified DC-stimulated LNMCs were added at 5×10^4, 1×10^4, and 5×10^3 cells/well. They were then incubated with 10^5 autologous vWR- (negative control) or vtetR-transduced papio-B (multiplicity of infection, 10). An additional negative control consisted of DC-stimulated LNMCs incubated in medium. Cells incubated in the presence of 25 ng of phorbol myristate acetate/ml–100 ng of ionomycin (Sigma)/ml were used as a positive control. Both mixed-cell populations were maintained overnight at 37°C and then washed and incubated for 2 h with the biotinylated MAb 7-B6-1 (anti-IFN-γ MAb; Mabtech) in phosphate-buffered saline (PBS) at 1 μg/ml. Wells were washed, and 0.5 U of streptavidin-bound alkaline phosphatase (AP; Boehringer Mannheim) in PBS was added for 90 min. The last step consisted in adding the BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium color substrate (Promega) for 1 h at room temperature. Spots were counted with an automated ELISPOT reader (Zeiss) by using KS-Elispot software. Specific responses against rtTA were considered positive if the number of spots exceeded the mean plus 3 standard deviations of cells incubated with control antigen-presenting cells (APC).

Histology. Hematoxylin and eosin staining was performed on paraffin-embedded tissue as described previously (8), and immunostaining was done on 6-μm-thick cryosections of snap-frozen tissue by using MAbs directed against human CD4 (M-T477 clone; Pharmingen), CD8 (SK1 clone; Becton Dickinson), and

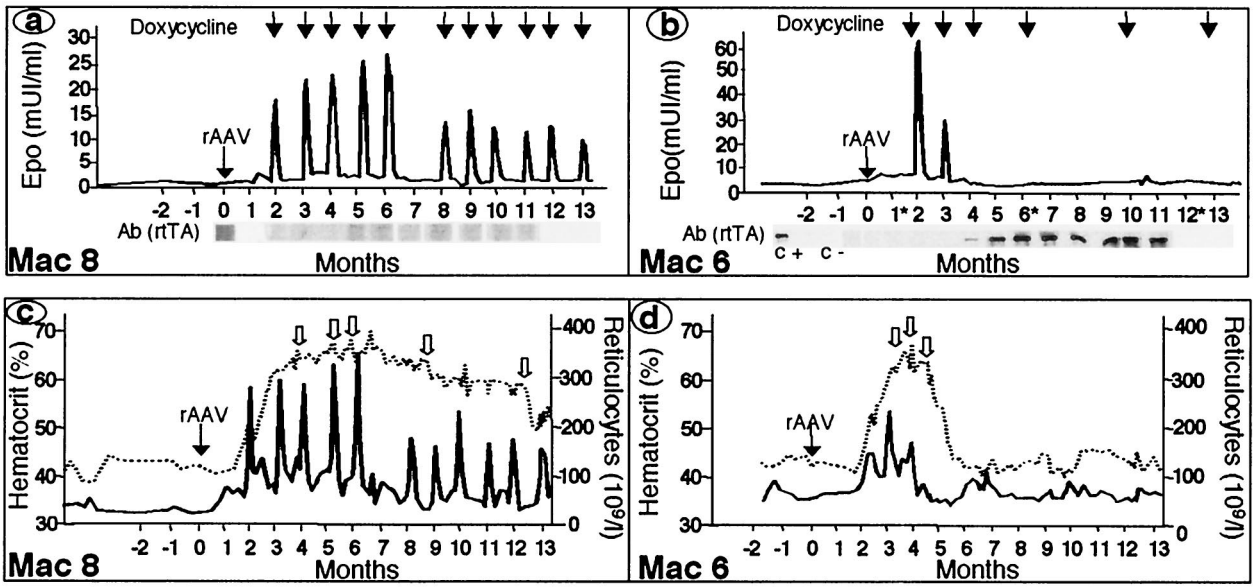

FIG. 2. Epo (a and b) and hematocrit (dotted lines) and reticulocyte (solid lines) (c and d) levels in representative animals Mac 6 and Mac 8. Solid arrows (a and b), DOX administration. Animals were occasionally bled (open arrows [c and d]). Ab (rtTA), detection of anti-rtTA antibodies in successive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

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-γ 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 rtTA 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

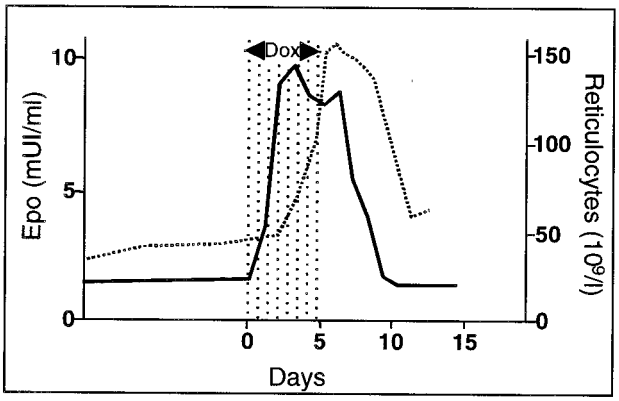

FIG. 3. Epo and reticulocyte levels, obtained from the last induction peak from Mac 8 (Fig. 2a) and determined on a daily basis upon DOX i.v. administration for 5 days (dotted area) and subsequent withdrawal.

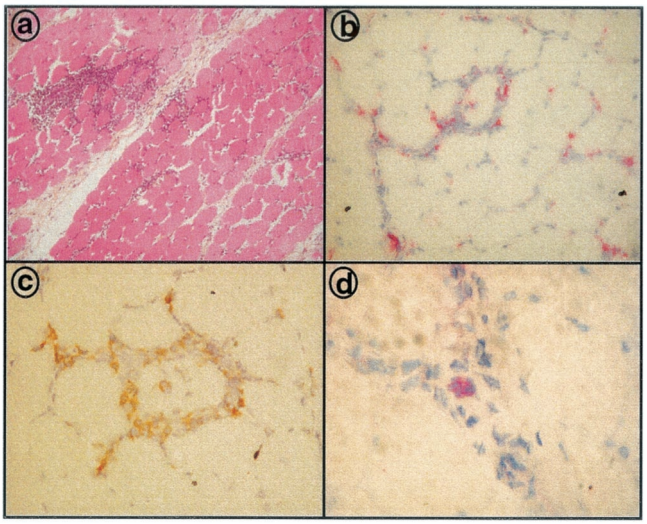

FIG. 4. Muscle biopsy sample obtained from Mac 6 6 months after rAAV injection. Shown is a phenotypic characterization of the cellular infiltrates. (a) Hematoxylin and eosin staining. Magnification, ×100. (b) Anti-CD68-AP. Magnification, ×200. (c) Anti-CD8-horseradish peroxidase. Magnification, ×300.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

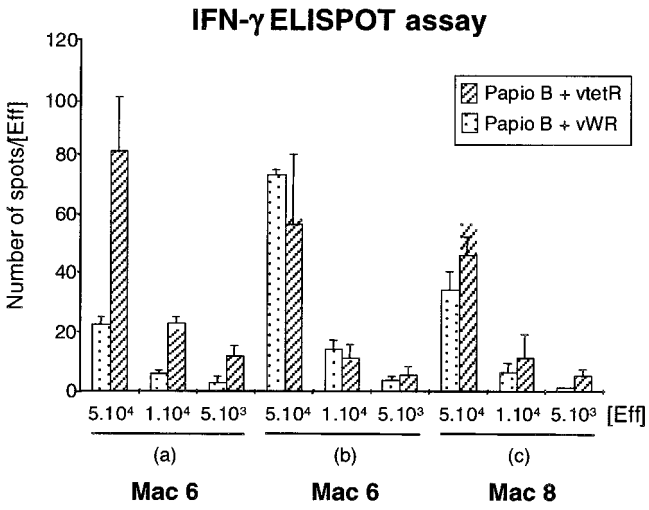

FIG. 5. Cytokine production can be measured at the single-cell level by using the ELISPOT technique, allowing calculation of T-cell frequencies. Shown is an IFN-γ ELISPOT assay using autologous LNMCs from Mac 6 and Mac 8, obtained from inguinal draining lymph nodes in the presence of autologous DCs expressing 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.

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.m. by 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 heterologous peptide. We incubated serum samples collected at different time intervals from each individual with purified tTA2(synth.)-His_6 peptide blotted onto nitrocellulose. Whereas no anti-rtTa antibodies could be detected in Mac 3 or Mac 8 (Fig. 2a), 1/100-diluted sera from Mac 4 through Mac 7 exhibited strong positivity against the transactivator protein (Fig. 2b). The positive control consisted of a commercial specific MAb against rtTA. Interestingly, there was a temporal correlation between the detection of the humoral immune response and the loss of Epo secretion upon DOX administration for Mac 4, Mac 5, Mac 6, and Mac 7 (Fig. 2b). Furthermore, Mac 5 and Mac 6 were subjected to biopsies at the site of vector administration at different times after vector administration. Control tissue was obtained from each individual and from an uninjected skeletal muscle area. The result showed that, whereas histology was normal 1 month after rAAV injection and at the time of sacrifice (12 months postinjection [p.i.]) (not shown), inflammation was detected in muscle samples obtained 6 months after vector injection (Fig. 4). Lymphomonocytic infiltrates associated with myofiber destruction were found in both Mac 5 and Mac 6 (Fig. 4a). Phenotypic analysis of the infiltrating cells showed the presence of $CD4^+$, $CD8^+$, and $CD68^+$ cells (Fig. 4b to d), concomitant with the detection of the humoral immune response (Fig. 2b). The simultaneous development of antibodies against the tetracycline-sensitive transactivator and the delayed and transient occurrence of cellular infiltrates at the sites of vector administration suggested that rtTA could be the target of an immune response in nonhuman primates. We further explored the cellular component of the rtTA-directed immune response by IFN-γ ELISPOT assays. LNMCs from inguinal draining lymph nodes were first stimulated for 10 days in the presence of autologous DCs expressing either the rtTA antigen (DC.rtTA) or one of the two control antigens, GFP (DC.GFP) or LacZ (DC.LacZ). Herpesvirus papio-transformed B lymphoblastoid cell lines (B-papio) were derived from PBMCs of Mac 3 through Mac 8. They were infected with either vaccinia virus vtetR (expressing the DNA binding domain of the rtTA) or control wild-type vaccinia virus vWR and subsequently used as stimulating cells for the IFN-γ ELISPOT assay. As shown Fig. 5, 16 months after vector administration, DC.rtTA-stimulated LNMCs from Mac 6 exhibited 81 ± 18, 23 ± 2, and 12 ± 4 IFN-γ spots per 5×10^4, 1×10^4, and 5×10^3 cells, respectively, when incubated in the presence of vtetR-transduced autologous B-papio as opposed to 22 ± 3, 6 ± 1, and 3 ± 2 IFN-γ spots per 5×10^4, 1×10^4, and 5×10^3 cells, respectively, obtained with DC.rtTA-stimulated LNMCs incubated in the presence of the control vWR-transduced autologous B-papio ($P < 0.05$). An additional control was obtained after incubation of DC.GFP-stimulated LNMCs with either vtetR- or vWR-transduced autologous B-papio, for which 57 ± 23, 11 ± 4, and 5 ± 3 IFN-γ spots versus 73 ± 1, 14 ± 3, and 4 ± 1 IFN-γ spots were counted per 5×10^4, 1×10^4, and 5×10^3 cells, respectively (*P*

> 0.1) (Fig. 5). In contrast, vtetR-transduced autologous B-papio from Mac 8 was unable to stimulate IFN-γ production from DC.rtTA-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 NH_2 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 *Bam*HI, 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 $\sim 10^9$ 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 concatemer 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

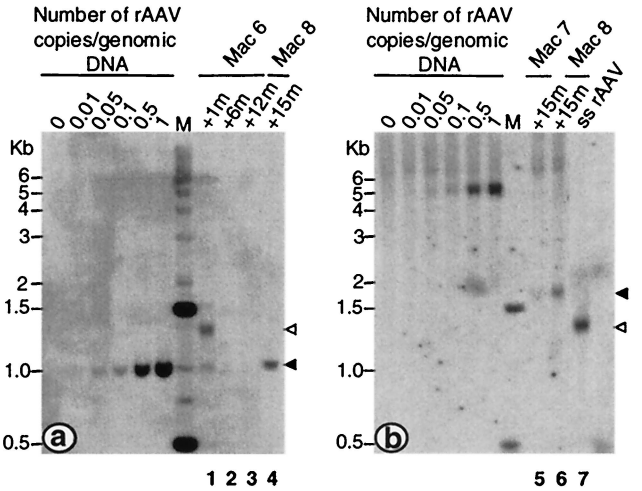

FIG. 6. Southern blot analysis of high-molecular-weight DNA extracted from muscles biopsy samples. (a) rtTA probe; (b) tetO-CMV probe. Muscle samples were obtained at +1, +6, and +12 months for Mac 6 (lanes 1 to 3) and at +15 months for Mac 7 (lane 5) and Mac 8 (lanes 4 and 6). Lane 7 corresponds to $\sim 10^9$ single-stranded vector genomes. Reference copy numbers correspond to high-molecular-weight DNA extracted from normal macaque muscle and run with plasmid DNA corresponding to 0.01 copy (0.25 pg) up to 1 copy (25 pg) of pAAVcm-ET(CAG). Open arrowheads, 1.4-kb single-stranded vector DNA input; filled arrowheads, 1-kb band (a, lanes 1 and 4) and ~1.8-kb band compatible with a head-to-tail concatemer (b). Quantification of vector signals relative to the reference copy number signals was done on a phosphorimager.

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 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

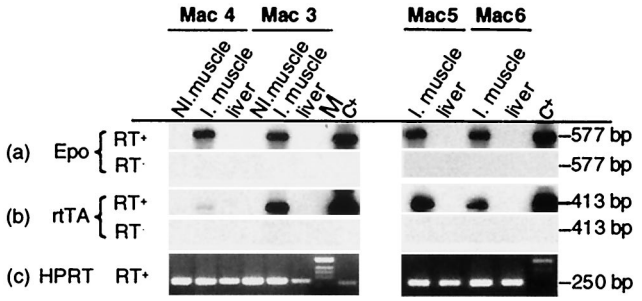

FIG. 7. Epo and rtTA RNA expression from rAAV-transduced muscles. Total RNA was extracted and processed as described in Materials and Methods. Samples were separated by electrophoresis, subsequently blotted under alkaline conditions (except for the HPRT control), and then hybridized with [^{32}P]dCTP random-primed labeled Epo (a) and rtTA (b) probes. (c) Control of RNA extraction. Lack of a DNA contaminant before reverse transcription is shown by the RT− samples. C$^+$, control PCR on 50 pg of AAV vector plasmid [pAAVcm-ET(CAG)]; NI. muscle, noninjected muscle; I. muscle, rAAV-injected muscle; M, marker.

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 monolymphocytic 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 APC 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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