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Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: Tolerization of factor IX and vector antigens allows for long-term expression

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ABSTRACT **Recombinant adenoviruses containing the canine factor IX (FIX) cDNA were directly introduced in the hind leg muscle of mice. We show that (*i*) in nude mice, high expression (1–5 μg/ml in plasma) of FIX protein can be detected for >300 days; (*ii*) in contrast, expression of FIX protein was transient (7–10 days) in normal mice; (*iii*) $CD8^+$ lymphocytes could be detected within 3 days in the infected muscle tissue; (*iv*) use of β_2-microglobulin and immunoglobulin M heavy chain "knockout" mice showed that lack of sustained expression of FIX protein is due to cell-mediated and humoral immune responses; (*v*) normal mice, once infected with recombinant adenovirus, could not be reinfected efficiently for at least 30 days due to neutralizing viral antibodies; and, finally, (*vi*) using immunosuppressive drugs, some normal mice can be tolerized to produce and secrete FIX protein for >5 months. We conclude that currently available adenoviral vectors have serious limitations for use for long-term gene therapy.**

Gene therapy holds a great promise for the treatment of many genetic diseases. Retroviral vectors have been extensively used to deliver genes into a wide variety of cell types but require *ex vivo* approaches because of their inability to infect postmitotic cells. In recent years much attention has been focused on a vector system that can deliver genes with high efficiency to a wide spectrum of nondividing cells *in vivo*. In particular, recombinant adenoviruses have been demonstrated to be very useful (1–5). Unfortunately, several studies with different gene products have revealed that following infection in a wide variety of target tissues, only transient expression is observed (6–12). Since the cells infected with the recombinant adenoviruses are rapidly eliminated, it is likely that the host immune system plays a major role in preventing sustained expression of the foreign genes. We have therefore undertaken a characterization of the specific immune responses and antigens involved in the lack of sustained expression by adenoviral factor IX (FIX) mediated gene therapy.

MATERIALS AND METHODS

Adenoviral Vectors. The canine FIX adenoviral vector AdMCdF9 was prepared as follows: a 2.5-kb fragment containing mouse muscle creatine kinase enhancer, cytomegalovirus enhancer/promoter, and dog FIX cDNA was excised from the retroviral vector LNMECIXL (13) and inserted into adenoviral plasmid pXCJL1 together with a 170-bp simian virus 40 poly(A) sequence. AdMCLacZ is identical to AdMCdF9 except that the canine FIX cDNA was replaced with *Escherichia coli* β-galactosidase (*lacZ*) gene. Both FIX and *lacZ* constructs in pXCJL1 were cotransfected with the pJM17 plasmid (14) into 293 cells (15), respectively, and recombinant adenoviral plaques were isolated and further purified by two rounds of plaque assays as described (16). The adenoviral vectors were propagated in 293 cells and purified by double CsCl banding as described (16). The purified virus was dialyzed against phosphate-buffered saline (PBS) and stored in aliquots with 15% glycerol at −80°C.

Animal Procedures. Adult Swiss Webster mice and nude (*nu/nu*) athymic mice were purchased from Harlan–Sprague–Dawley. β_2-Microglobulin knockout, β_2m(−/−), mice (17) and IgM heavy chain (μ chain) knockout, μMT/μMT mice (18) were kindly provided by Leonard Shultz (The Jackson Laboratory). About 1×10^9 plaque-forming units (pfu) of purified AdMCdF9 or AdMCLacZ virus was diluted into 100 μl of PBS and injected into muscles of both hind legs of anesthetized adult mice at 6–10 weeks of age (5–10 μl per site, 50 μl per leg). Plasma was taken by bleeding the tail vein at the indicated times and canine FIX in mouse plasma was determined by ELISA (19). For immunosuppression, 50 mg of cyclophosphamide (CyP) per kg (Adria Laboratories) and 10 mg of cyclosporine A (CsA) per kg (Sandoz Pharmaceutical) were given i.p. three times every 2 weeks for 8 weeks.

Histology and Immunocytochemistry. Muscles from hind legs of injected mice were used in morphology and immunolabeling studies. For morphological observation, the muscle was fixed in Bouin's fixative overnight, embedded in paraffin, sectioned, and stained in hematoxylin/eosin. For immunolabeling procedures, the muscles were frozen in freezing medium without prior chemical fixation. Cryostat sections were cut and thawed, and the labeling steps were carried out at room temperature. The sections were blocked in 10% normal goat serum for 30 min, washed three times with PBS, and incubated with rat anti-mouse CD8 antibodies (Abs) [purified IgG from 53-6.72 hybridoma (American Type Culture Collection) ascites] for 30–60 min. Biotinylated rabbit anti-rat IgG (Vector Laboratories) was used as the secondary Ab and the sections were incubated with avidin-biotin peroxidase complex (ABC kit; Vector Laboratories) and visualized with diaminobenzidine. All sections were counterstained with paraicolaou stain "EA-65."

Western Blot Analysis. Purified canine FIX or purified AdMCdF9 virions were subjected to SDS/PAGE and then transferred onto Immobillon-P membrane (Millipore). The membrane was blocked with 5% non-fat milk in PBS and cut into strips. Each strip was incubated with 1:60 diluted plasma at different time points from AdMCdF9 injected mice or with

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Abbreviations: FIX, factor IX; β_2m, β_2-microglobulin; Ab, antibody(ies); pfu, plaque-forming units; RT–PCR, reverse transcription–polymerase chain reaction; CTL, cytotoxic T lymphocyte; CsA, cyclosporine A; CyP, cyclophosphamide; CMI, cell-mediated immunity.

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1:500 diluted mouse anti-human FIX monoclonal Ab (Boehringer) and followed by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG and 5-bromo-1-chloro-3-indolyl phosphate/nitroblue tetrazolium developer (Promega).

RESULTS

FIX Expression in Infected Mice: Cellular and Humoral Immunity. Therapeutic levels of FIX (5 μg/ml) in mouse plasma could be detected following infection with 10^9 pfu of recombinant virus in the muscle tissue of nude mice, which gradually declined to 1 μg/ml over a period of 1 year (Fig. 1*A*). Even though adenoviral DNA does not integrate in the host chromosome, the slow "turnover" of muscle cells allows the sustained expression for extended periods. However, when the same FIX adenoviruses were injected in normal mice, expression of FIX (ranging from 0.1 to 1 μg/ml) declined precipitously within days to basal level (Fig. 1*B*). Because canine FIX expressed in mice is antigenic, the lack of FIX expression may be due to the presence of FIX Ab in the mouse plasma (20). Indeed, FIX Ab can be detected by day 13 (see Fig. 4*A*). To further investigate the possibility that production of FIX Ab may be responsible for the lack of measurable FIX protein in the plasma, we infected the recombinant viruses in μMT/μMT mice. Once again, expression of FIX protein declined (Fig. 1*C*) even though no FIX Ab were detectable (see Fig. 4*C*). We presumed that the loss of FIX expression could, in addition, be due to cytotoxic T lymphocyte (CTL) response against the viral antigens and, perhaps, FIX protein. We therefore injected recombinant adenoviruses into β_2m(−/−) mice (Fig. 1*D*). Expression of FIX protein declined rapidly, perhaps due to Ab to FIX protein (Fig. 1*D*; see Fig. 4*C*). From these results we conclude that recombinant adenoviral vectors introduced in mouse muscle elicit cellular and humoral immune response.

To confirm the cellular immune response to recombinant adenoviruses, we performed histology on the infected tissues. Fig. 2 shows migration of lymphocytes in muscle within 24 hr of infection (Fig. 2*B*). The presence of $CD8^+$ cells was confirmed by staining with CD8-specific Ab (Fig. 2*E*). Infiltration of lymphocytes could be detected in μMT/μMT (Fig. 2*F*) but not in β_2m(−/−) mice (Fig. 2*G*). Thus, it appears that rapid cellular immune response may lead to elimination of infected muscle cells leading to loss of FIX expression. The presence of $CD8^+$ lymphocytes and dependence on β_2m suggested that the CTL responses are major histocompatibility complex class I restricted, which requires *de novo* protein synthesis. The use of E1A defective adenoviruses to construct AdMCdF9 implied lack of viral replication, but at high multiplicity of infection the requirement for E1A can be overcome by some cellular factor (21). We therefore tested to see if mRNAs for late viral proteins are synthesized in infected cells, and data obtained with reverse transcription–polymerase chain reaction (RT-PCR) in Fig. 3 shows that mRNA for hexon is detectable in muscle cells 4 days after infection.

To confirm that Ab against adenoviral proteins are generated, we performed Western blot analysis on the serum obtained from infected mice. Fig. 4*A* shows that FIX Ab can be detected in mice at day 13 following infection and levels remain high thereafter for at least 43 days. No FIX Ab were detectable in μMT/μMT mice (Fig. 4*C*) but could be readily detected in β_2m(−/−) mice (Fig. 4*C*). In addition to the Ab to canine FIX, extensive humoral responses to adenoviral antigens were also detected in infected animals. Specifically, Ab against viral hexon and fiber protein could be readily detected (Fig. 4*B*).

Reinfection with Recombinant Adenoviruses. Since adenoviruses do not integrate, it is important to know if the animals can be reinfected, once the expression levels diminish. However, the presence of the host immune response in the infected animals suggests that reinfection may not be productive or efficient. We therefore reinfected mice that were previously infected for 30 days with either recombinant FIX adenovirus or with recombinant *lacZ*-β-gal adenovirus. Results in Fig. 5 show that 1 day after reinfection in Swiss Webster mice, vector (*lacZ* adenovirus) primed animals express <20% of factor IX produced in the naive mice, while animals primed with FIX adenovirus barely express detectable amounts of FIX. When similar experiments were carried out in μMT/μMT mice incapable of generating Ab, about 60% of the activity could be detected when the animals were primed with *lacZ* adenovirus. We infer from these results that the loss of activity is due to CTL response to viral antigens. These data are predicted by the extensive lymphocyte infiltration in the muscle of adenovirus

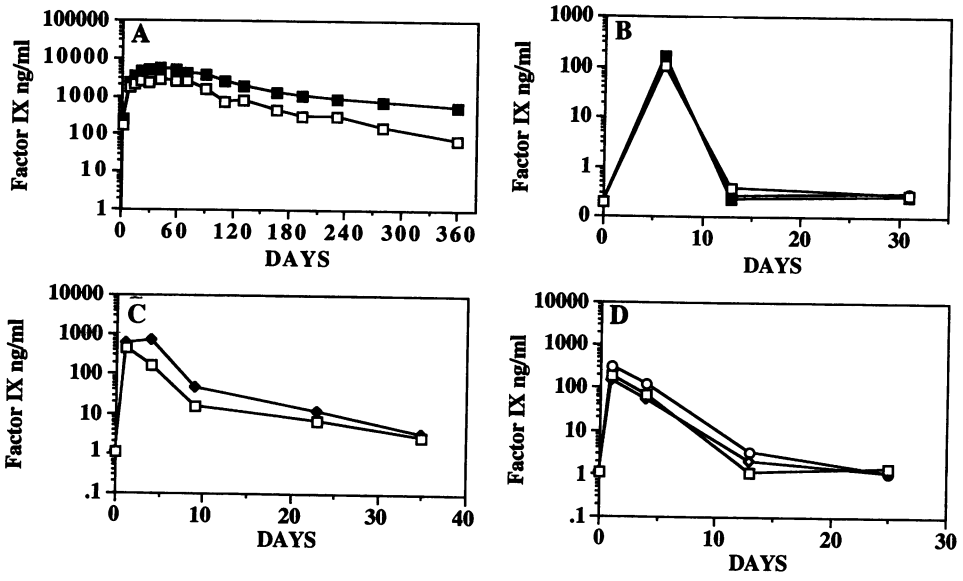

FIG. 1. *In vivo* FIX expression mediated by AdMCdF9 in normal and immune compromised mice. Nude (*A*), Swiss Webster (*B*), μMT/μMT (*C*), and β_2m(−/−) (*D*) mice were injected with 1×10^9 pfu of AdMCdF9. Plasma FIX levels from mice were determined by ELISA at the indicated time following AdMCdF9 administration. Each line represents a different mouse. Two additional nude mice injected with AdMCdF9 have produced FIX proteins for >250 days, while four more infected nude mice have produced FIX protein for >150 days.

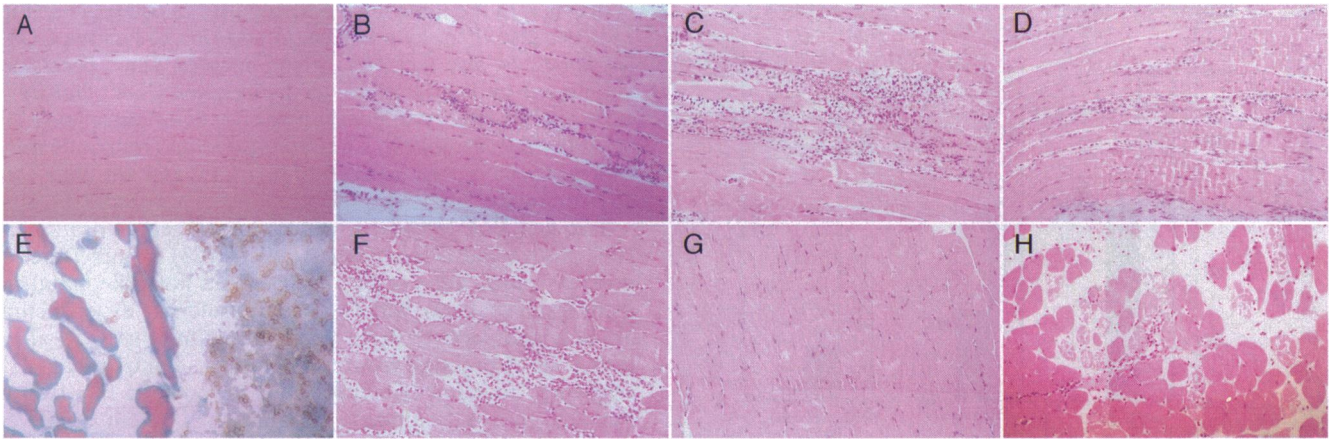

FIG. 2. Lymphocyte infiltration at the injection site. Histology sections taken from the hind legs of Swiss Webster (*A–E* and *H*), μMT/μMT (*F*), and β_2m(−/−) mice (*G*); control (*A*), 1 day (*B*), 3 days (*C* and *F*), 5 days (*G*), or 10 days (*D*) after AdMCdF injection and staining with hematoxylin/eosin. The presence of $CD8^+$ lymphocytes at the injection site can be seen with immunohistochemistry (*E*). Priming of Swiss Webster mice with AdMCdF9 results in greater lymphocyte infiltration 1 day after second injection (*H*). Also notice extensive tissue damage in *D* and *H*. (*A–D* and *F–H*, ×20; *E*, ×40.)

primed mice (Fig. 2*H*). Surprisingly, an additional 50% of FIX expression was lost in the μMT/μMT mice as a result of priming with FIX adenovirus. Our interpretation is that CTLs are generated against the FIX protein itself and are killing the FIX-producing cells. The role of humoral responses upon recombinant adenoviral infection was further underscored by low FIX levels in β_2m(−/−) mice.

Effects of CsA and CyP. The preceding data suggest that a major limitation of successful gene therapy with recombinant adenoviruses is the host immune system. We therefore opted to tolerize the host to the adenoviral and FIX proteins. We used CsA and CyP, two commonly used immunosuppressants, with the hope that CsA would block cell-mediated immunity (CMI), while CyP would act by killing dividing cells (22) and, as such, could eliminate CMI and humoral responses. Fig. 5*B* shows that CsA alone had no effect while CyP alone and in combination with CsA allowed expression to last longer after initial decline. In one mouse, CyP treatment resulted in complete tolerance and FIX levels were essentially unchanged for >150 days.

DISCUSSION

Correction of genetic defects would require technology that allows long-term expression of the therapeutic gene product. To date, retroviral vectors have partially fulfilled this requirement by integrating in the host genome, but, unfortunately, sustained expression at therapeutic levels has not been attained. Furthermore, inability of retroviruses to integrate in postmitotic cells obligates that cells from tissues like liver, muscle, or fibroblasts are propagated *in vitro*, transduced, and then transplanted into the animal. This *"ex vivo"* approach is not only cumbersome but also requires efficient transplantation techniques.

Recombinant adenoviruses containing foreign genes can be generated at high titers and have been extensively used for *in vivo* gene delivery. Two major limitations of adenoviral vectors were immediately apparent: (*i*) episomal nature of the viral DNA and (*ii*) likely humoral and cellular response to viral antigens. Here we show that host immune responses are very effective in eliminating infected cells and preventing reinfection. The CMI was confirmed by (*i*) rapid migration of $CD8^+$ lymphocytes at the site of injection (Fig. 2 *B–E*), (*ii*) lack of lymphocyte infiltration in β_2m(−/−) mice (Fig. 2*F*), and (*iii*) loss of FIX expression in μMT/μMT mice. These data are in general agreement with those reported by Yang *et al.* (23). We have further shown that despite the use of E1A deficient adenoviral vectors, late viral gene expression occurs in infected

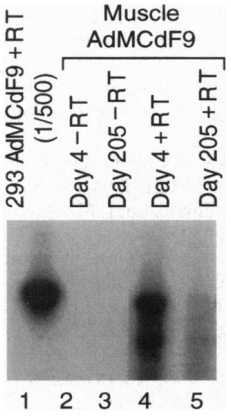

FIG. 3. RT–PCR analysis of late viral RNA. Total cellular RNA was isolated from hind leg muscle of nude mice 4 days and 205 days after AdMCdF9 infection, treated with RNase-free DNase, and analyzed for hexon RNA by RT–PCR. The hexon sequence in the RT–PCR products was confirmed by Southern analysis with an adenovirus 5 hexon-specific probe. Diluted RT–PCR product (1:500) from AdMCdF9 infected 293 cells was used as a positive control.

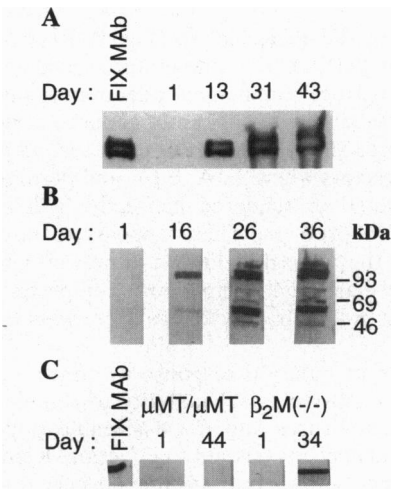

FIG. 4. Neutralizing Ab to FIX and adenovirus proteins. Sera from Swiss Webster mice (*A* and *B*) or μMT/μMT and β_2m(−/−) mice (*C*) injected with 1×10^9 AdMCdF9 were used to probe FIX (*A* and *C*) and adenovirus (*B*) protein blots.

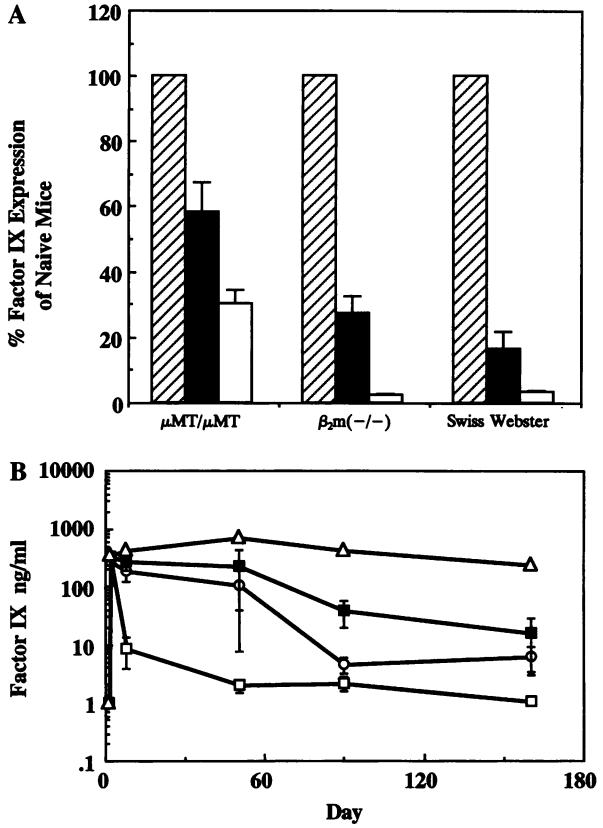

FIG. 5. (*A*) Comparison of *in vivo* FIX expression mediated by AdMCdF9 in normal and immune compromised mice following a second injection. AdMCdF9 (□) and AdMCLacZ (■) injected Swiss Webster, μMT/μMT, and β_2m(−/−) mice were given 1×10^9 AdMCdF9 as a second injection ≈4–6 weeks later. Plasma FIX levels from mice 1 day after the second injection were determined by ELISA. Due to the different sizes and body weights of the different strains of mice, data are presented as the percent of FIX expressed in the unprimed mice (▨), which was 102 ng/ml, 135 ng/ml, and 183 ng/ml for the Swiss Webster, β_2m(−/−), and μMT/μMT mice, respectively. Data represent the results of several independent experiments. (*B*) Effects of CsA and CyP on AdMCdF9 gene therapy. Swiss Webster mice were given either CsA (□) or CyP (○) following AdMCdF9 administration. Data represent the combined results of two groups of mice that were bled on days 1, 8, 50, and 90 or on days 1, 9, 58, and 94. Each time point is the average of three mice, with the exception of the nonrepresentative CyP tolerized mouse (△). ■, CsA plus CyP.

cells as judged by RT–PCR analysis (Fig. 3). Recent results by Engelhardt *et al.* (24), where a temperature-sensitive E2A gene was used to construct recombinant adenovirus, suggests that CTL response to viral antigens can be reduced at nonpermissive temperature. Thus it is hopeful that a second generation of adenoviral vectors where E1A, E2A, and perhaps E4 gene have been deleted or rendered ineffective will be able to reduce the CMI problem. There appears to be a general consensus that the E3 region, known to prevent viral antigen presentation by major histocompatibility complexes I and II, is useful to retain in the viral vectors as it prevents CTL killing of target cells.

The problem of humoral response to viral antigens presents two major obstacles: (*i*) inability to reinfect with recombinant adenoviruses and (*ii*) a sizeable population is seropositive and may be resistant to infection. Reinfection of μMT/μMT mice shows that in the absence of humoral response, >60% levels of FIX protein can be detected. The lack of full expression is likely due to the presence of CTLs (Fig. 2*H*). Although we have not tried to reinfect nude mice, it is likely that reinfection will be possible. The long-term expression in the nude mice as compared to β_2m(−/−) (Fig. 1) is because the athymic mice, in addition to lacking $CD8^+$ lymphocytes, also lack $CD4^+$ lymphocytes. Since B-cell development and antibody diversity require $CD4^+$ helper T cells, the possibility of generating effective neutralizing Ab in nude mice is unlikely. Therefore the data from nude mice represent the *in vivo* expression in a CTL and antibody-free environment.

The fact that long-term expression of secreted antigens (this study) and nonsecreted antigens (23) in nude mice is possible raises interest in tolerizing the host. Neonatal injections have been widely used to tolerize experimental animals and recently have been successfully used to allow long-term expression from recombinant adenoviruses (25). However, neonatal injections have never been reported in humans, and it is unlikely that this will be an approved practice for human gene therapy. Therefore, we explored the effectiveness of widely used immunosuppressive drugs (22). CsA had no effect on FIX expression, a result anticipated since CsA is not a potent inhibitor of humoral immunity. The increase in FIX expression in animals treated with CyP is most likely due to this drug's ability to eliminate CMI and humoral response. Although only one in four mice was completely tolerant, this demonstrates that adult animals can be tolerized to allow long-term gene therapy and breeds optimism in that, although the host immune system remains a major obstacle, future research may be able to remove this barrier.

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