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Transient immunomodulation with anti-CD40 ligand antibody and CTLA4Ig enhances persistence and secondary adenovirus-mediated gene transfer into mouse liver

(hepatic gene therapy/costimulation/T cells/human alpha 1-antitrypsin/factor IX)

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ABSTRACT **Although recombinant adenovirus vectors offer a very efficient means by which to transfer genetic information into cells *in vivo*, antigen-dependent immunity limits the duration of gene expression and prevents retreatment. Recombinant murine CTLA4Ig and anti-CD40 ligand antibody block costimulatory interactions between T cells and antigen presenting cells. We previously reported that murine CTLA4Ig prolongs adenoviral-mediated gene transfer, but does not allow for secondary expression after readministration of the vector. In studies described here, when anti-CD40 ligand and recombinant murine CTLA4Ig were coadministered around the time of primary vector administration (*i*) prolonged adenovirus-mediated gene expression (length of experiment up to 1 year) from the livers of >90% of treated mice was observed, and (*ii*) secondary adenovirus-mediated gene transfer was achieved in >50% of the mice even after the immunosuppressive effects of these agents were no longer present. Nearly two-thirds of these mice had persistent secondary gene expression lasting for at least 200–300 days. Neither agent alone allowed transduction after secondary vector administration. Treated mice had decreased immune responses to the vector as shown by markedly decreased production of neutralizing antibodies, diminished spleen proliferation responses and IFN-γ production *in vitro*, and reduced T cell infiltrates in the liver. These results suggest that it may be possible to obtain persistence as well as secondary adenoviral-mediated gene transfer with transient immunosuppressive therapies.**

The use of recombinant adenovirus vectors for gene transfer induces an immune response directed against the vector, vector-transduced cells, and, in some cases, the transduced gene product. Data from several groups (1–4) using murine models of gene transfer to the lung and the liver have helped to elucidate the nature of this immune response. In summary, it appears that T cell dependent, antigen-specific immunity limits the duration of transduced gene expression following inoculation of naive animals and largely prevents gene expression following secondary administration (5–7).

One approach to enhance adenoviral-mediated gene transfer is to modulate or block the host immune response. Immunosuppression with cyclosporin alone has not enhanced adenoviral-mediated gene therapy (6, 7). Gene expression was prolonged with combined and continuous cyclosporin and cyclophosphamide therapy, but secondary vector administration was not attempted (6). FK506 alone prolonged gene expression when administered daily for the duration of the experiment but did not fully block the development of neutralizing antibody; this suggests that secondary vector administration, though not evaluated, would have been impaired (8). In addition to their limited efficacy and toxicity, these regimens would likely also impair pre-existing immunity to other microbes. We reasoned that to be acceptable for use in human gene therapy a regimen that (*i*) resulted only in transient immunosuppression, (*ii*) was not cytoablative, (*iii*) minimized effects on pre-existing immunity, and (*iv*) did not result in immunological tolerance to wild-type adenovirus was needed and we have sought to develop such an approach.

Antigen-presenting cells (APCs) process and present protein antigens to T cells in association with major histocompatibility complex (MHC) molecules. The complex of processed antigenic peptides and MHC provides one signal needed for T cell activation. B7–2 and B7–1 proteins (CD86 and CD80, respectively) are present on efficient APCs and bind to CD28 on T cells, thereby providing a second, costimulatory signal, which is particularly important for the primary response of naïve T cells to novel antigens (9–13). A second ligand for B7–1 and B7–2 that is expressed on activated T cells is CTLA4, which primarily dampens T cell activation (14, 15). A soluble molecule composed of the extracellular domain of CTLA4 fused to an immunoglobulin IgGFc domain (CTLA4Ig) binds B7 ligands with 20-fold greater affinity than CD-28 (16, 17), thereby blocking the binding of CD28 to B7–1 or B7–2 and inhibiting T cell priming. Moreover, engagement of the T cell receptor by antigen–MHC in the absence of this costimulatory signal commonly produces T cell anergy or prolonged unresponsiveness (16) and may result in prolonged survival of allografted organs (18, 19). Our initial studies (20) examined the effects of treatment with soluble, recombinant murine CTLA4Ig (muCTLA4Ig) with a first generation adenovirus vector (21). In control mice, hAAT expression declined more than 100-fold between 2 and 7 weeks after administration, whereas in 11 of 13 mice treated with muCTLA4Ig expression persisted for the duration of the experiment (5–6 months). These results are comparable to those observed (3, 5, 7) in genetically immunodeficient mice.

Activated T cells also express cell surface proteins that stimulate APC and B cell function. A critical T cell protein in this regard is the CD40 ligand. This protein is expressed primarily on activated CD4 T cells and is critical to their ability to provide help for B cell antibody responses (22–24). CD40 ligand also enhances APC function, in part by inducing or enhancing expression of the B7 proteins (reviewed in refs. 25 and 26). The importance of this ligand is underscored by the X-linked hyper IgM syndrome (McKusick no. 308230), which is the consequence of a genetic

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Abbreviations: APC, antigen-presenting cell; MHC, major histocompatibility complex; hAAT, human alpha 1-antitrypsin; cFIX, canine factor IX; pfu, plaque-forming units; IFN-γ interferon-γ.

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deficiency in expression of a functional CD40 ligand (27). In mice, administration of mAb to CD40 ligand (MR1) reproduces the defects of the genetic immunodeficiency; it profoundly inhibits antibody production (especially antibody other than IgM), reduces the rate and efficiency with which antigen–specific T cell responses develop (26), and inhibits a variety of responses that are dependent on T cell-mediated immunity (e.g., graft vs. host disease) (22, 28). Blockade of the CD40 pathway enhances adenoviral-mediated gene expression, but the effect is incomplete (29). However, the simultaneous blockage of both CD40 and CD28 costimulatory pathways with MR1 and CTLA4Ig has recently been shown to improve the engraftment of highly immunogenic allografts (30). Reported below are results of experiments using murine CTLA4Ig or MR1 alone or in combination. These data indicate that dual blockade of these bidirectional costimulatory interactions between T cells and B cells/APCs enhances adenoviral-mediated gene transfer and fulfills many of the requirements for an acceptable adjunct as outlined above.

MATERIALS AND METHODS

Animal Studies. BALB/c and C3H/HeJ female mice were purchased from The Jackson Laboratory, and studies were performed in accordance with the institutional guidelines set forth by the University of Washington. All animals were housed in SPF facilities. General experimental procedures with the mice were done as described (20).

Reagents to Block Costimulatory Interactions. Murine CTLA4Ig (muCTLA4Ig) and the control mAb L6 were prepared and used as described (20). MR1, an mAb to CD40 ligand, is a purified hamster IgG antibody as previously described (31).

Virus Preparations. The Ad/RSVhAAT, Ad/PGKhAAT (21), and Ad/RSVcFIX (32) are first generation adenovirus type 5, E1a deleted and partial E1b, E3 deleted recombinant vectors transducing human alpha 1-antitrypsin (hAAT) and canine factor IX (cFIX), respectively. They were prepared and assayed for the presence of RCA as described (5).

Biological Assays. Antibody determinations, spleen cell proliferation, cytokine measurements, immunohistochemistry, muCTLA4Ig, and hAAT assays have been described (20). MR1 concentrations in serum were assayed by capture ELISA. Briefly, ELISA plates (Nunc) were coated overnight with a mAb to murine CD8 (mAb 53–6). After blocking with PBS plus 3% BSA and washing, a recombinant fusion protein of murine CD40 ligand and murine CD8 was added, plates were washed, and then serial dilutions of serum samples were added. After incubation plates were washed, incubated with peroxidase-conjugated goat anti-hamster IgG (Jackson ImmnoResearch), washed, and developed with ABTS (Kirkegaard & Perry Laboratories). A standard curve was generated using purified MR1. All ELISA determinations were made in duplicate at each time point tested.

Bacteriophage ϕX174 was prepared and administered to mice as described (20). Anti-phage neutralizing antibody titers and the fraction of antibodies that were IgG were determined as described (33).

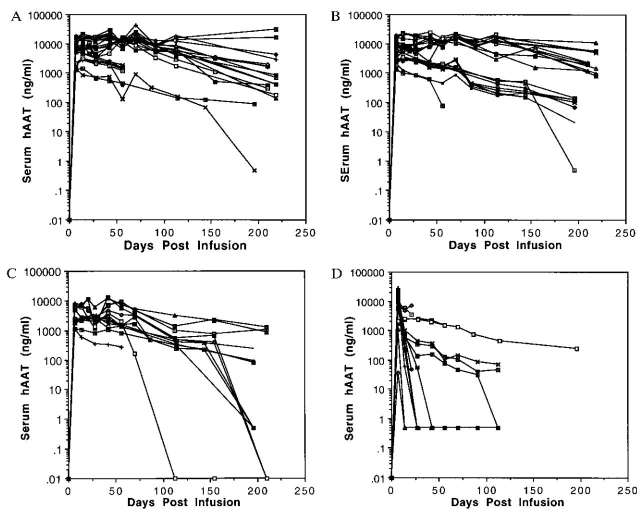

FIG. 1. Adenovirus-mediated hAAT expression in mice treated with (*A*) muCTLA4Ig, $n = 27$; (*B*) muCTLA4Ig/MR1, $n = 25$; (*C*) MR1, $n = 15$; (*D*) L6, $n = 16$. C3H or BALB/c mice were injected with 5×10^9 plaque-forming units of Ad/RSVhAAT or Ad/PGK hAAT by tail vein on day 0. The dose of muCTLA4Ig was 200 μg on days 0, 2, and 10; the dose of MR1 was 250 μg on days 0, 2, 4, and 6; and the dose of L6 was 200 μg on days 0, 2, and 10 or 0, 2, 4, and 6. Serum samples analyzed for hAAT were tested at least in duplicate. Each line represents an individual animal.

RESULTS

The Effects of CTLA4Ig and MR1 on Persistence of Gene Expression. We initially compared the efficacy of MR1 (anti-CD40 ligand), muCTLA4Ig, or the combination in prolonging hAAT expression after adenovirus-mediated gene transfer to the liver. Three independent experiments were performed using BALB/c or C3H/HeJ mice. The results in these experiments and with both mouse strains were comparable, so the data were pooled (Fig. 1). Persistence of hAAT expression in muCTLA4Ig alone treated mice (Fig. 1*A*) was similar to that previously reported (20) and to that in mice given both muCLTA4Ig and MR1 (Fig. 1*B*); more than 90% of the mice had persistent high-level (less than a 10-fold decline from the peak level) expression of hAAT for at least 180 days, and in many expression persisted at high levels for more than 1 year (data not shown). This pattern of expression was similar to that reported by us and others in *scid* mice (5). Most of the mice receiving MR1 alone also had persistent high-level expression, although the fraction (10 of 15) was somewhat lower than in mice receiving muCTLA4Ig alone or in combination with MR1 (Fig. 1*C*). In marked contrast, all but one of the 16 L6-treated control animals had a two-log or greater decrease in expression by 2–7 weeks (Fig. 1*D*).

Humoral Immunity and Secondary Gene Transfer. Our previous studies demonstrated that CTLA4Ig administration alone significantly, but not completely, attenuated the humoral immune response to the vector. Despite this, the low levels of anti-adenoviral neutralizing antibodies present were sufficient to inhibit secondary gene transfer (20). Compared with L6-treated control animals, each of the treatment regimens reduced production of anti-adenoviral neutralizing antibodies (Table 1). Treatment with MR1 alone was the least consistently effective, since more than one-half of these mice had detectable antibodies by 8–10 weeks after vector infusion (Table 1, experiments 1 and 2). In contrast, 18 of 22 mice given muCTLA4Ig alone and only 3 of 27 mice given both MR1 and muCTLA4Ig-treated mice had detectable anti-adenoviral neutralizing antibodies within the first 8–10 weeks (Table 1, experiments 1–3). By 16–18 weeks, all of the mice given either MR1 or muCTLA4Ig had neutralizing antibodies but only 3 of the 15 mice receiving both had detectable antibodies (Table 1 experiments 1 and 3). All of the control mice had high titers of anti-adenoviral neutralizing antibodies within 2 weeks of adenovirus administration.

To determine if inhibition of the antibody response with any of these regimens would allow gene expression from a second adenoviral vector, mice in one of these experiments (Table 1, experiment 2) were given Ad/RSVcFIX 8 weeks after the primary vector administration. These mice also received doses of the same agents given with the primary vector on days 0, 2, and 10 relative to the time of secondary vector administration. None of the control mice or the mice that received MR1 or muCTLA4Ig alone (not shown) produced detectable amounts of cFIX. In contrast, 4 of 6 mice receiving both muCTLA4Ig and MR1 had substantial expression of cFIX, all but one mouse had a two-log decline in gene expression over a period of 30–40 days as seen in naive controls (Fig. 2*A*). All of these animals developed detectable

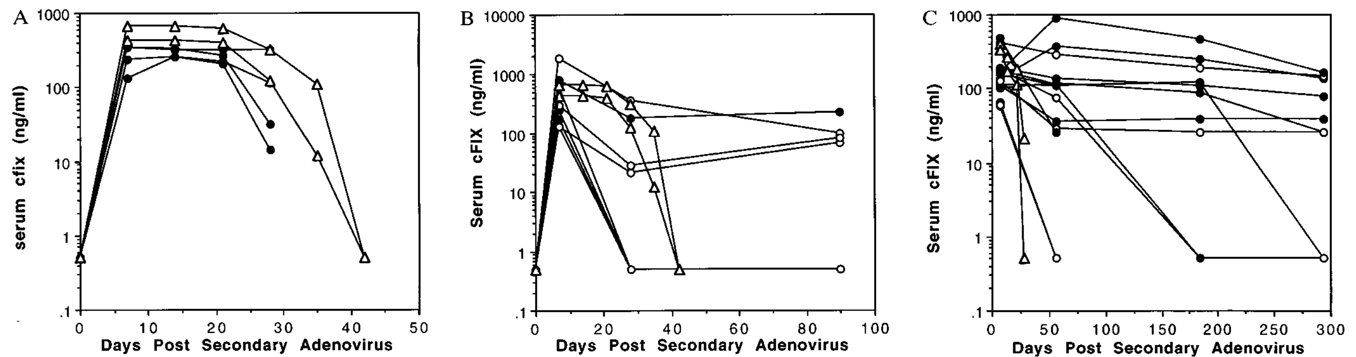

FIG. 2. Secondary adenovirus-mediated hepatic gene transfer. C3H/HeJ or BALB/c mice were injected with 5×10^9 plaque-forming units (pfu) of Ad/PGKhAAT (*A*) or Ad/RSVhAAT (*B* and *C*) by tail vein on day 0. Mice in *A* and *B* received immunomodulatory agents as described in Fig. 1 and the mice in *C* received CTLA4Ig/MR1 or L6 on days −1, 0, 2, 10, 17, and 24 relative to adenovirus administration. The mice were redosed with 5×10^9 pfu of Ad/RSVcFIX: (*A*) 8 weeks after primary adenovirus infusion, $n = 6$; (*B*) 16 weeks after primary infusion, $n = 9$; (*C*) 18 weeks after primary infusion, $n = 29$. The mice received a second dose of immunomodulatory agents (●) or L6 control protein (○) at the time of Ad/RSVcFIX adenovirus admininistration on the same schedule as they received the first time. The mice designated by Δ represent naive animals that were infused with the same dose of Ad/RSVcFIX. Periodic serum samples were assayed for cFIX by ELISA in duplicate. The experiments shown correspond to experiments 2, 3, and 4 outlined in Table 1. Only mice that express cFIX after Ad/RSVcFIX administration are shown.

antibodies by 2 weeks after secondary vector administration (Table 1, experiment 2).

In the next experiment (experiment 3), animals initially received Ad/RSVhAAT with muCTLA4Ig alone (not shown) or with MR1 and then after 16 weeks received Ad/RSVcFIX. A portion of these animals received a second dose of these immunomodulatory agents at the time of Ad/RSVcFIX injection (Fig. 2*B*). Eight of 9 mice receiving both agents showed some level of secondary transduction. Although four of these animals had only transient expression (detectable for 2–3 weeks) of relatively low levels of cFIX, four animals had persistent gene expression for at least 3 months, the length of the experiment (Fig. 2*B*). The four mice that received CTLA4Ig and MR1 at the time of Ad/RSVcFIX administration did not have greater persistence of cFIX expression than those that did not. Nonetheless, administration of a second dose of CTLA4Ig/MR1 with a secondary vector decreased the titer of antibodies directed against the adenovirus. In fact, one of these four animals had no detectable antibodies 8 weeks after the second and 24 weeks after the first vector administration (Table 1, experiment 3).

To determine if increased dosing of these immunomodulatory agents would be more consistently effective, another experiment was performed in which mice received muCTLA4Ig and MR1 on days −1, 0, 2, 10, 17, and 24, relative to the time of administration of the primary vector, Ad/RSVhAAT. As in the previous experiments, >90% of mice receiving the combination of MR1 and muCTLA4Ig showed persistent, high-level hAAT expression (data not shown). The anti-adenoviral neutralizing antibody titers are shown in Table 1, experiment 4. Similar to mice receiving fewer doses of both agents (Table 1, experiments 1–3), only 9 of 34 mice developed detectable neutralizing antibodies by 16 weeks. Consistent with this, following administration of Ad/RSVcFIX at 18 weeks, 15 of 29 (52%) mice expressed factor IX. Ten of these 15 mice (67%) had persistent expression for at least 2 months, and 8 mice had persistent gene expression for at least 200–300 days (Fig. 2*C*). The expression and persistence of cFIX expression did not differ between those mice that received additional doses of MR1 and muCTLA4Ig (given on the same schedule as with primary vector administration) at the time of second vector administration (Fig. 2*C*). Ten of 21 mice that did receive secondary immunomodulatory therapy and 5 of 8 mice that did not expressed cFIX. In addition, 3 of 10 mice with persistent cFIX expression did not receive additional immunomodulation therapy. In contrast to experiment 3 (Table 1), in which three doses of muCTLA4Ig were given, and in which all but one of the mice developed anti-adenoviral neutralizing antibodies (Table 1), 6 of 15 mice (40%) in experiment 4 did not develop neutralizing antibodies by 4 weeks after the administration of the second vector. This suggests that readministration of immunomodulatory agents will likely be required if repetitive doses of recombinant adenoviruses are to be given.

Host Immune Status at the Time of Secondary Transduction. Adjunct immune modulation strategies, such as the one described here, would be of greatest clinical utility if sustained expression and readministration of adenovirus can be achieved without continuous immunosuppression. To determine the immunologic status of the mice at the time they were given the second adenoviral vector (Ad/RSVcFIX), we assayed serum for CTLA4Ig and for MR1 and determined their response to immunization with the T-dependent neoantigen bacteriophage ϕX174.

Adenovirus-mediated gene expression persisted in the treated mice beyond the period when concentrations of muCTLA4Ig and MR1 declined to levels below those affecting the immune response. Serum muCTLA4Ig concentrations declined to 1 μg/ml by 6 weeks and were undetectable by 8 weeks. Serum MR1 concentrations varied between 2,500–10,000 ng/ml during the first 4 weeks. In the mice receiving MR1 alone, concentrations of MR1 declined to values <5 ng/ml by 1–2 months. In mice receiving both muCTLA4Ig and MR1, clearance of MR1 was delayed in some, so that concentrations by 90 days ranged from <65–1,476 ng/ml. In experiment 4, where additional doses of the immunomodulatory agents were given, concentrations of muCTLA4Ig declined in a manner similar to those animals given only 3 doses: in 21 of 26 mice there was no detectable MR1 (<5 ng/ml) and in 3 mice up to 200 ng/ml was present in the serum at 16–18 weeks.

To determine the immunologic status of the animals at the time they were given a second dose of adenovirus, the T-dependent antibody response to bacteriophage ϕX174 was determined (Table 2) at the time of second vector administration in experiments 3 and 4 (Table 1, Fig. 2 *B* and *C*). Previous studies have shown that T cell dependent antibody responses are inhibited by muCTLA4Ig (34) and depend on CD40 ligand function (22). Thus, this test is a sensitive measure of immunologic competence. The titers, expressed as Kv (rate of phage inactivation over time) are shown in Table 2.

In experiment 3, animals were infused with phage at the time of secondary Ad/RSVcFIX administration. The animals that received immunomodulatory agents only at the time of initial vector administration had a robust response with normal IgM to IgG switching, similar to that seen in the L6 treated controls. In contrast, animals receiving a second dose of immunomodulatory agents (either CTLA4Ig alone or CTLA4Ig/MR1) at the time of Ad/RSVcFIX and phage administration had depressed primary and secondary antibody responses and IgG class switching. A similar pattern of response was seen in experiment 4 (Table 2). A normal robust primary and secondary response with IgG class

Table 1. Neutralizing antibody titers

Exp.	Treatment	Week 2	Week 4	Week 8	Week 10	Week 16	Week 18	Week 22/24
1	Combined (n = 5)	<16 (<16)	<16 (<16)		<16 (<16–←16)	64 (<16–64)		
	CTLA4Ig (n = 5)	<16 (<16–16)	←16 (<16–16)		←16 (←16–64)	32 (32–512)		
	MR1 (n = 11)	16 (<16–64)	16 (←16–64)		32 (16–64)	128 (64–1028)		
	L6 (cont) (n = 5)	64 (64–256)	128 (64–256)		64 (32–256)	512 (512–1024)		
2	Combined (n = 12)		<16 (<16–←16)	<16 (<16–←16)	16 (←16–64)*			
	CTLA4Ig (n = 8)		←16 (←16)	←16 (←16)	ND			
	MR1 (n = 10)		←16 (←16–16)	←16 (←16–16)	ND			
	L6 (cont) (n = 10)		1024 (1024–>1024)	1024 (1024)	1024 (512–>1024)†			
3	Combined (n = 10)		<16 (<16)		<16 (<16)	<16 (<16–←16)		(a) 1024 (1024)‡ (b) 16 (>16−16)
	CTLA4Ig (n = 9)		←16 (<16–16)		16 (<16–64)	64 (←16–1024)		1024 (1024)
	L6 (cont) (n = 10)		256 (64–1024)		512 (256–1024)	1024 (1024)		>1024 (>1024)
4	Combined (n = 34)		<16 (<16–16)		<16 (<16–640)		<16 (<16–128)	(a) 256 (64–1024)§ (b) ←16 (<16−1024)¶
	L6 (cont) (n = 14)		512 (256–1024)		1024 (1024)		1024 (1024)	

Neutralizing antibody titers in mice receiving muCTLA4Ig, MR1, or both (combined) or L6 (control). In experiments 1, 2 and 3 the mice received muCTLA4Ig on days 0, 2, 10 and MR1 on days 0, 2, 4 and 6 relative to Ad/RSVhAAT (experiments 1 and 3) or Ad/PGKhAAT (experiment 2) administration. The mice in experiment 4 received muCTLA4Ig/MR1 on days −1, 0, 2, 10, 17, and 24 relative to infusion of Ad/RSVhAAT. The titers are expressed as the median reciprocal (1/x) dilution yielding 75% neutralization with the range in parentheses. ND, not determined. The lowest dilution of serum tested was 1:16. Values listed as ←16 indicate that antibodies were detected but resulted in <75% neutralization at a 1:16 dilution. Experiments 2, 3, 4 are equivalent to those in Fig. 2 *A*, *B*, and *C*, respectively. Note: †, n = 2; ¶, n = 21.

*Mice received an infusion of Ad/RSVcFIX (secondary adenovirus) at 8 weeks relative to the infusion of Ad/PGKhAAT. These mice received muCTLA4Ig/MR1 or L6 on the same dosing schedule as the first administration at 8 weeks at the time of secondary adenovirus administration (n = 6).

‡Mice received Ad/RSVcFIX (secondary adenovirus) at 16 weeks relative to Ad/RSVhAAT administration. Mice received L6 (a) or (b) muCTLA4Ig/MR1 at the time of secondary adenovirus administration (16 weeks) on the same schedule as the first administration (n = 5).

§Mice received Ad/RSVcFIX (secondary adenovirus) at 18 weeks relative to the first adenovirus infusion. Mice received L6 (a) or (b) muCTLA4Ig/MR1 at the time of secondary adenovirus administration (18 weeks) on the same dosing schedule as the first administration (n = 9).

switching was observed in L6 controls and animals that had received one dose of immunomodulatory agents 18 weeks before. Similar to experiment three, the response against the phage was blocked in animals getting a second dose of CTLA4Ig/MR1. Taken together, these results show that at the time of secondary adenovirus administration, in both experiments 3 and 4, the mice had normal T and B cell function, as determined by their responses to bacteriophage.

Effects of Combined muCTLA4Ig and MR1 on the Cellular Immune Response. We previously reported that treatment of mice with muCTLA4Ig alone attenuated but did not block the development of T lymphocyte responses to the vector as assessed by proliferation and IFN-γ production (20). To determine the effects of combined treatment of mice with muCTLA4Ig and MR1, the response of splenocytes to the adenoviral vector was assessed (Fig. 3). In mice treated with muCTLA4Ig and MR1 neither proliferation (Fig. 3) nor IFN-γ (not shown) production in response to the Ad/RSVhAAT vector differed significantly at any time point from results with cells from naive mice that had not been exposed to adenovirus. In contrast, mice that received Ad/RSVhAAT and the control mAb L6 mounted a brisk response to the vector. The responses with cells from muCTLA4Ig plus MR1-treated and L6-treated mice differed significantly for proliferation at days 11 and 28 ($P < 0.05$). Results for IFN-γ production paralleled those for proliferation, but the difference was significant only at day 11 ($P < 0.05$). Furthermore, an antigen-specific response was consistently observed in cultures of splenocytes from the L6 controls, in that each of three mice produced detectable amounts of IFN-γ (>200 pg/ml) at each time point, whereas IFN-γ was never detected in splenocyte cultures from naive mice. Treatment with muCTLA4Ig plus MR1 did not completely ablate responses to the vector, since IFN-γ was detectable above background in cultures from one of three mice at each of the three time points. The inhibition of IFN-γ production and proliferation in the muCTLA4Ig plus MR1-treated mice did not appear to reflect a shift from a T-helper 1- to T-helper 2-type immune response, since interleukin-4 was not detected in the supernatants of splenocytes stimulated with virus under any condition.

Immunohistological Analysis. Animals used for splenocyte assays were also evaluated for cellular infiltrates in the liver (Table 3). A similar approach used in our earlier studies in CTLA4Ig treated animals showed decreased $CD3^+$, $CD4^+$, and $CD8^+$ cells

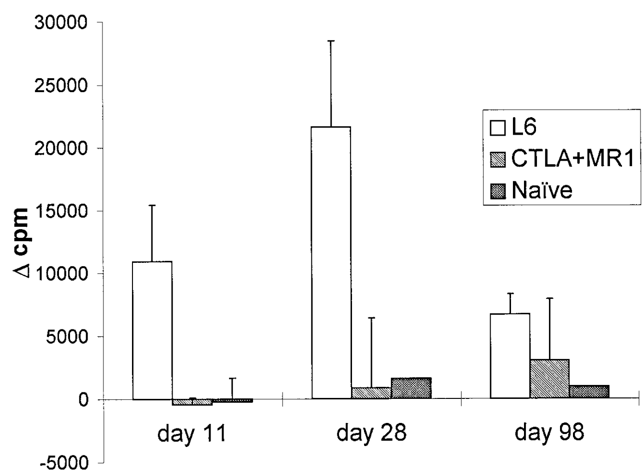

FIG. 3. Splenocyte proliferation assays. Proliferation by splenocytes in response to Ad/.RSVhAAT. At the indicated days after administration of Ad/.RSVhAAT, splenocyte responses were assessed as described in the *Materials and Methods*. Results shown are proliferation in response to UV-inactivated Ad/RSVhAAT at the highest concentration of UV inactivated virus tested (10^8 pfu/ml), which was optimal. [^{3}H]Thymidine uptake from unstimulated and anti-CD3-stimulated cells was similar in each group at each time point.

Table 2. ϕX174 antibody responses

Treatment scheme	Primary immunization	Secondary immunization	IgG, %
Experiment 3			
Combined initially and combined at the time of phage administration (n = 4)	0.515 (0.3–0.9)	6.84 (4.9–11.2)	8 ± 7
Combined initially but not at the time of phage administration (n = 5)	1.33 (0.6–4.3)	107 (104–324)	100 ± 0
muCTLA4Ig initially and muCTLA4Ig at the time of phage administration (n = 5)	0.310 (0.25–0.5)	9.9 (6.2–30.4)	31 ± 25
muCTLA4Ig initially but not at the time of phage administration (n = 5)	1.45 (0.3–5.6)	111 (14–572)	97 ± 5
Control (L6) (n = 10)	1.2 (0.36–2.6)	86 (18–266)	97 ± 5
Experiment 4			
Combined initially and combined at the time of phage administration (n = 21)	0.306 (0.11–0.95)	0.278 (0.028–2.05)	*
Combined initially but not at the time of phage administration (n = 11)	6.4 (3–13)	62.4 (4.5–184)	95 ± 13
Control (L6) (n = 13)	6.4 (0.5–16.7)	37 (6.5–103)	98 ± 4.6

Phage inactivation titers in mice from experiments 3 and 4 (see Table 1) immunized with bacteriophage ϕX174. Phage was injected i.v. in mice at 16 and 18 weeks in experiments 3 and 4, respectively, relative to primary (Ad/RSVhAAT) adenovirus administration. The values represent the geometric mean expressed as the Kv or rate of phage inactivation. The range is listed in parentheses. The percent of IgG is expressed as mean ± SD. The titer after primary immunization represents 2 weeks after phage infusion. The titer after secondary immunization was 2 weeks and 3 weeks after second phage infusion for experiments 3 and 4, respectively.

*The total concentration of immunoglobulin directed agains the phage was so low that the percentage of IgG was too low for an accurate assessment.

between days 11 and 28 after adenovirus administration (20). Animals given adenovirus and then CTLA4Ig/MR1 or L6 (controls) were analyzed for the relative quantities of $CD3^+$ (T cells), $CD4^+$, $CD8^+$, and MHC class II+ cells on days 11, 28, and 98 after adenovirus infusion compared with naive mice (Table 3). CTLA4Ig/MR1 nearly completely blocked the infiltrate of $CD3^+$, $CD4^+$, $CD8^+$, and induction of class II MHC expression on days 11 and 28 compared with L6-treated controls that had relatively large infiltrates containing all four cell types. At day 98, animals treated with adenovirus and L6 had a decrease in the cellular infiltrate. However, all cell types were still present in numbers above that seen in naive animals. Small numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were seen in some of the muCTLA4Ig/MR1 mice at day 98. Together with the antibody and *in vitro* splenocyte response data, these results suggest that CTLA4Ig/MR1 significantly attenuates but does not completely block the immunologic response or induce tolerance to the vector.

DISCUSSION

The current studies extend previous work by ourselves and others, seeking to enhance adenoviral vector-mediated gene transfer to the liver with transient and focused modulation of the immune response. In previous studies (20), we found that brief administration of muCTLA4Ig alone, which would block the CD28/B7 costimulatory pathway, resulted in prolonged expression of the transduced gene product. This therapy attenuated but did not ablate the cellular or humoral immune response to the vector, and secondary vector administration was precluded. This study corroborates these results. It also indicates that administration of the anti-CD40L mAb (MR1) alone, which blocks the interaction between CD40 and CD40 ligand and the second major T cell–B cell/APC costimulatory pathway, produces similar results. However, MR1 was somewhat less effective than muCTLA4Ig in prolonging primary vector-transduced hAAT expression. In contrast to the results with either agent alone, the combination not only results in persistence of primary gene expression, but allows for effective secondary adenovirus vector-transduced gene expression in a majority of mice. Primary gene expression persisted in 90% of mice and secondary gene expression persisted in two-thirds of mice transduced a second time. Importantly, secondary gene transfer occurred in animals that had intact immunologic responses to a T dependent antigen, and persistence was not dependent on additional immunosuppressive therapy. This suggests that prolonged generalized immunosuppression is not necessarily required for persistence or secondary gene transfer.

Recent studies by Yang *et al.* (29) demonstrated that anti-CD40 ligand alone was sufficient to prolong adenovirus-mediated β-galactosidase expression for at least 1 month, although there was a 2-fold drop in the number of transduced cells over a 25-day period. Also, on secondary vector administration, only 8% of the hepatocytes were transduced compared with 90% in naive controls; the persistence of secondary gene expression was not evaluated. In contrast to the studies by Yang *et al.* (29), the immunomodulation strategy used here resulted in gene expression after secondary vector administration that was equivalent to that seen in naive mice, and expression persisted in most of the animals. Furthermore, the results from our studies suggest that immunosuppressive concentrations of anti-CD40 ligand may still have been present at 1 month, when gene expression was assessed and secondary vector was administered in the studies of Yang *et al.* (29). Our results are consistent with those of Yang *et al.* (29) in that at 2–4 weeks after MR1 administration alone, most of the animals still have no or very low levels of neutralizing antibodies directed against the adenovirus. If secondary adenovirus administration was performed at 4 weeks after MR1 administration, gene transfer may have also been possible in our animals. However, by the time the

Table 3. Cellular infiltrates

	Day 11				Day 28				Day 98			
	CD3	CD4	CD8	Cl II	CD3	CD4	CD8	Cl II	CD3	CD4	CD8	Cl II
comb	0.3 (0.3)	0.1 (0.1)	0 (0)	0.4 (0.1)	0 (0)	0 (0)	0.1 (0.1)	0.1 (0.1)	1.2 (0.6)	0.3 (0.3)	0.1 (0.1)	0 (0)
L6	2.8 (0.4)	2.0 (0.4)	3.0 (0)	2.8 (0.2)	2.2 (0.6)	1.4 (0)	2.4 (0.3)	1.3 (0.4)	1.2 (0.4)	0.3 (0.2)	0.9 (0.4)	0.4 (0.6)

Immunohistochemical staining for $CD3^+$, $CD4^+$, $CD8^+$, MHC class II was performed on the same mice described in Fig. 3. Naive animals were analyzed at the same time. The sections were scored using a 0–4 scale (20) to grade portal inflammation. The mean values are given within each experimental group (n = 3) and the standard deviations are given in parentheses.

serum MR1 concentrations declined to subtherapeutic or nondetectable levels, none of the mice given MR1 alone were transduced a second time with the current studies, and MR1 was less effective when used alone than CTLA4Ig in blunting the titer of anti-adenovirus antibodies.

It does not appear that a state of true immunologic tolerance was achieved by any of the treatment regimens. Splenocyte responses were above the background level detected in naive mice, and by 90 days, a low-grade T cell infiltrate was detected in the livers of CTLA4Ig/MR1 animals. Furthermore, if these compounds were not given at the time of secondary vector administration at 16 or 18 weeks, anti-adenoviral neutralizing antibodies eventually developed. However, we cannot exclude the possibility that a smaller subset of the animals, which received two doses of adenovirus, were redosed with CTLA4Ig/MR1, and continued to have no detectable neutralizing antibodies, were truly tolerant. These results suggest that the ability to achieve more than two transduction events with systemic adenovirus administration may be limited. Nonetheless, long-term persistence of gene expression after primary and secondary administration was seen in a substantial number of animals, suggesting that the need for repeated administration may be sufficiently infrequent to be acceptable. Currently, we cannot predict how the greater than 300 days of persistent expression achieved in mice will translate into other species, including humans.

There has been recent discussion and debate about the role of the transgene product in the ultimate elimination of gene expression (35, 36). The issue is complicated by the fact that the adenovirus may function as an adjuvant. Also, the immunologic mechanisms leading to the loss of transgene product are not clear and may be different with various transgene products and in different mouse strains. In BALB/c, C3H/HeJ, and C57BL/6 mice, the reduction in hAAT gene expression correlates with the loss or persistence of adenoviral DNA in the liver (unpublished results). Nevertheless, whatever the mechanism(s), adenovirus-mediated gene expression can be maintained in mice using the immunomodulatory therapy described in this study. Studies to determine if this is also true in primates are underway.

The other general strategy to enhance gene transfer is to produce a less antigenic vector alone or in combination with immunomodulatory therapy. In theory, this has advantages over pharmacologic interference with the immune system. However, it is not clear whether or not the elimination of other vector genes will result in a vector that will persist in the absence of an immune response. In fact, the elimination of most of the vector genome produces a vector that is equally able to transduce cells *in vitro* and *in vivo* compared with first generation E1a deleted vectors, yet the DNA of the deleted vector is unstable in transduced cells (37). Ultimately, if adenoviral vectors are to be useful for disorders requiring persistent gene expression, it will be crucial to determine the minimal essential vector genes/sequences needed and to identify appropriate immunomodulatory therapy, which together result in persistent transgene expression and/or allow for repetitive vector administration.

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