Repeated Administration of Adenoviral Vectors in Lungs of Human CD4 Transgenic Mice Treated with a Nondepleting CD4 Antibody

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J Immunol 1999; 163:448-455;
http://www.jimmunol.org/content/163/1/448
Repeated Administration of Adenoviral Vectors in Lungs of Human CD4 Transgenic Mice Treated with a Nondepleting CD4 Antibody

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The central role of CD4+ T cells in regulation of adenovirus vector-mediated immune responses has been documented previously in murine models. We analyzed the effects of a nondepleting mAb to human CD4 (CD4 mAb; Clenoliximab) on immune functions following intratracheal administration of adenoviral vectors in murine CD4-deficient mice (muCD4KO) expressing a human CD4 transgene (HuCD4 mice). Treatment of HuCD4 mice with Clenoliximab inhibited both cell-mediated and humoral immune responses to adenoviral Ags. Chronic treatment of HuCD4 mice with Clenoliximab permitted successful readministration of adenoviral vectors at least four times. The ability to readminister these vectors is associated with marked suppression of neutralizing Ab responses to viral capsid proteins. Clenoliximab also inhibited CTL and prolonged expression of the transgene. T or B cell responses to adenovirus did not emerge after the effects of a short course of Clenoliximab diminished. These data illustrate the potential utility of a nondepleting CD4 Ab in facilitating gene therapy using adenoviral vectors. The Journal of Immunology, 1999, 163: 448–455.

R ecombinant adenoviral vectors, rendered defective by deletion of immediate early genes E1a and E1b, show promise as vehicles for somatic gene therapies (1, 2). The tropism of these vectors for airway epithelia is being exploited in the development of gene therapies for lung diseases, e.g., cystic fibrosis (3, 4). One hurdle in the application of recombinant vectors is the loss of transgene expression as a result of elimination of transduced bronchoepithelial cells by infiltrating cytotoxic T cells (5). Viral proteins expressed from the first generation E1-deleted adenoviral vectors have been shown to be targets for CTL activation (6). Another problem is the diminished efficiency of a second vector administration. In vivo administration of E1-deleted adenovirus vectors to the lung results in activation of Th cells, which trigger generation of neutralizing Abs that prevent vector readministration. Several studies in murine models of lung-directed gene therapies have confirmed the critical role of CD4+ T cells in helper responses that facilitate the induction of CTL as well (7, 8).

Numerous model systems have used various in vivo systems to evaluate the importance of Th cells on Ag-specific immune functions (9, 10). The key role of CD4+ T cell activation was shown in prevention of experimental autoimmune encephalomyelitis, type II collagen-induced arthritis, allograft tolerance, and virus-induced pathology (11). We have been interested in evaluating adenovirus vector-induced immune responses to determine their role in the persistence of transgene expression and the ability to readminister these vectors for gene therapy. Previous studies have indicated that depletion of CD4+ T cell with anti-CD4 Ab, GK1.5 resulted in persistent transgene expression and efficient ability to readminister vector in mice (7). However, depletion of CD4+ T cells is not desired in human clinical trials. In this study we have evaluated the efficacy of a nondepleting anti-human CD4 Ab.

The CD4 molecule is a nonpolymorphic membrane glycoprotein of 55 kDa that consists of four extracellular domains (D1–D4). This extracellular domain of murine CD4 molecules shows 55% sequence homology with its human counterpart (12). Residues at positions 19, 89, and 165 on the human CD4 molecule are critical for interaction with MHC class II molecules in both human and mice. Interspecies CD4/MHC class II interactions can mediate functional immune responses (13–16). It has also been demonstrated that human CD4 can interact with mouse MHC class II molecules and signal through mouse p56lck(17, 18). Killeen et al. (13) have demonstrated that the human CD4 transgene reconstitutes, phenotypically and functionally, the CD4+ T cell compartment in murine CD4-deficient mice (muCD4KO). The current study used these HuCD4 mice to evaluate the immune-suppressive effects of a nondepleting mAb to human CD4 in adenoviral vector-mediated immune responses to the lung.

Materials and Methods

Animals

Mice deficient for the murine CD4 gene (muCD4KO mice) and murCD4KO mice transgenic for human CD4 (HuCD4) were bred in a specific pathogen-free facility under contract at Charles River (Wilmington, MA). HuCD4 mice have been previously described (13). Briefly, (C57BL/6 × SJL/J)F1 eggs fertilized by (129/SV × C57BL/6 × DBA2) males were used for microinjection by crossing of male founder to (C57BL/6 × 12/SV) females. These mice were subsequently bred to homozygocity on the H2d locus.

Abbreviations used in this paper: muCD4KO, murine CD4 knockout; HuCD4, human CD4; GFP, green fluorescent protein; BAL, bronchoalveolar lavage; SEB, Staphylococcus enterotoxin B; CF, cystic fibrosis; D1, domain 1.
Recombinant adenoviruses

The construction of E1-deleted recombinant adenoviruses expressing Escherichia coli β-galactosidase (H5.010CBlacZ, henceforth called Ad-lacZ), human alkaline phosphatase (H5.100CBALP, henceforth called Ad-ALP), green fluorescent protein (H5.000CMVGFP, henceforth called Ad-GFP), and luciferase (H5.000CMVLuc, henceforth called Ad-Luc) were amplified on 293 cells and purified on cesium chloride gradients as described previously (19, 20).

Antibodies

Clenoliximab, a Primatized mAb to human CD4, contains the variable heavy chain dimer formation and reduces Fc receptor binding and (M. Reddy et al., manuscript in preparation). This mAb has no C1q binding or complement-fixing activity and has a dramatically reduced Fc receptor binding activity. The preparation of purified Clenoliximab used in this study was provided by the Department of Pharmaceutical Technologies, SmithKline Beecham Pharmaceuticals (King of Prussia, PA). In one experiment a human IgG1 isotype Ab of irrelevant specificity was used as a control.

Study design

Particles of each vector (1 × 10^11) were instilled intratracheally in a volume of 50 µl. For this purpose, animals were anesthetized, and a skin incision was made on the ventral surface of the neck. Muscles were separated to expose the trachea into which 50 µl of vector was instilled using a 27-gauge needle. The skin incision was then closed using a 4-0 vicryl suture material. Clenoliximab was administered i.p. to the mice intratracheally as follows: vector 1, lacZ; vector 2, ALP; vector 3, GFP; and vector 4, Luc. The arrows indicate i.p. treatment with the CD4 Ab Clenoliximab, and the shaded rectangles indicate days of necropsy. Groups 1–4 are controls in which naive animals were treated with vector in the absence of Clenoliximab, followed by treatment with a subsequent vector 30 days later and necropsy 4 days after each vector administration. Group 5 represents chronic treatment with Clenoliximab concurrent with four sequential administrations of vector. Group 6 received a short course of Clenoliximab at the time of vector 1 administration followed by a second vector (i.e., GFP) 90 days later.

Neutralizing Ab assays

Neutralizing Ab titers were measured by analyzing the ability of serum or bronchoalveolar lavage (BAL) fluid to inhibit transduction of reporter virus, Ad-GFP, into HeLa cells. Various dilutions of serum/BAL were pre-incubated with reporter virus for 1 h at 37°C and added to 90% confluent HeLa cell cultures. Cells were incubated for 16 h. Expression of GFP was measured by fluorescence imaging (Molecular Dynamics, Sunnyvale, CA). The neutralizing titer of Ab was calculated by the highest dilution of the sera at which 50% of the cells turned green.

Adenovirus-specific Igs

Serum (diluted 1/200) and BAL (diluted 1/20) samples from animals were analyzed for adenovirus-specific isotype specific Igs (IgM, IgG1, IgG2a, and IgA) by ELISA. For the ELISA, 96-well flat-bottom, high binding Immulon-IV plates were coated with 200 µl of Ad-LacZ Ag (5 × 10^10 particles/ml) in PBS overnight at 4°C, washed four times in PBS containing 0.05% Tween, and blocked in PBS with 1% BSA for 2 h at 4°C. Appropriately diluted samples were added to Ag-coated plates and incubated overnight. Plates were washed four times in PBS with 0.5% Tween and incubated with biotin-conjugated rat anti-mouse IgM, IgG1, IgG2a, or IgA (1/2000 dilution; PharMingen, San Diego, CA) for 2 h at 4°C. Plates were washed as described above, and a 1/20,000 dilution of peroxidase-conjugated streptavidin was added. Color development was induced by addition of ASBT substrate (Kirkegaard & Perry, Gaithersburg, MD). OD values were read at 405 nm on a MRX Dynatech Microplate reader (Dynatech, Chantilly, VA).

Lymphoproliferative assays

Spleenocytes from mice were obtained at various time points during the study. Mouse spleenocytes were disaggregated to a single-cell suspension made on a wire mesh that is passed through a nylon filter. Triplicate cultures of 100 µl of 1 × 10^6 cells/ml lymphocytes were cultured with a multiplicity of infection of 10 of inactivated Ad-lacZ, 100 ng/ml Staphylococcus enterotoxin B (SEB), or medium alone. Proliferation was measured by a 16-h [3H]thymidine (1 μCi/well) pulse. Results are expressed as stimulation index, which is a ratio of the counts per minute of Ag/SEB-stimulated cultures per counts per minute of cultures in medium alone.

Cytokine release assays

Spleenocytes were cultured with or without Ag (i.e., inactivated Ad-lacZ at a multiplicity of infection of 10) for 48 h in a 24-well plate. Cell-free supernatants were collected and analyzed for the presence of IL-2, IL-4, IFN-γ, and IL-10 by ELISA as described previously (21).

FACS analysis

Heparinized blood obtained from individual mice was stained with OKT4A-FITC, OKT4-PE (Ortho Diagnostics, Raritan, NJ), and murine CD8-CyC (PharMingen, San Diego, CA) or with isotype controls for 10
particles of Ad-lacZ, IFN-γ, IL-4, and IL-10 were measured in serum by ELISA and plotted as picograms per milliliter. The results were obtained from pooled spleens of three to five mice, and the values shown are the mean ± 1 SD from three or four separate experiments. Supernatants were analyzed from cells treated with medium (○) or stimulated with Ad-lacZ (●). These analyses were performed with μCD4KO, HuCD4, or HuCD4 treated with Clenoliximab (CD4 mAb). * p < 0.001, using Student’s t test.

FIGURE 2. Cytokine secretion profile. Animals were administered 1 × 10^11 particles of Ad-lacZ intratracheally in the presence or the absence of Clenoliximab (CD4 mAb), and splenocytes were harvested 14 days later for stimulation with adenovirus and analysis for secretion of cytokines. IL-2, IFN-γ, IL-4, and IL-10 were measured in serum by ELISA and plotted as picograms per milliliter. The results were obtained from pooled spleens of three to five mice, and the values shown are the mean ± 1 SD from three or four separate experiments. Supernatants were analyzed from cells treated with medium (○) or stimulated with Ad-lacZ (●). These analyses were performed with μCD4KO, HuCD4, or HuCD4 treated with Clenoliximab (CD4 mAb). * p < 0.001, using Student’s t test.

FIGURE 3. CTL responses. The experimental groups evaluated for CTLs are described in Fig. 2. A, μCD4KO; B, HuCD4; C, HuCD4+CD4Ab. Splenocytes from three to five mice, harvested on day 11, were cultured in the presence of 1 multiplicity of infection of Ad-lacZ for 5 days and used as effector cells. Specific lysis was analyzed on mock-infected (●) or Ad-lacZ-infected (○) P815 target cells in a 51Cr release assay. The percent specific lysis is expressed as a function of different E:T cell ratios. The results are the mean and SEM of two separate experiments in the μCD4KO mouse and three separate experiments in the HuCD4 mouse.
Experimental protocols were established to evaluate the potential of Clenoliximab as an adjunct to vector in the long term treatment of cystic fibrosis (CF). A summary of the study groups is presented in Fig. 1. Group 5 received chronic doses of Clenoliximab during a regimen of four sequential vector administrations spaced 1 mo apart. All vectors were E1-deleted adenoviruses, although each expressed a different reporter gene, to easily distinguish them from one another (vector 1, lacZ; vector 2, ALP; vector 3, GFP; vector 4, Luc). Groups 1–4 represent treatment with each vector of naive animals without Clenoliximab and treatment with the next vector 30 days later; animals were euthanized, and tissues were harvested for transgene expression 4 days after each vector administration. Group 6 was designed to evaluate the durability of transient Clenoliximab treatment by following the animals long after the initial Ab effect diminished (i.e., study readministration with GFP vector 90 days after treatment with lacZ vector and short course of Clenoliximab).

Groups 5 (chronic treatment) and 6 (short course) were evaluated for the effect of Clenoliximab on peripheral CD4 and CD8 T cells as detected by flow cytometry with mAbs specific to CD4 (OKT4A-FITC and OKT4-PE) and CD8 (CD8-CyC; see Fig. 5). OKT4A binding to an epitope within domain 1 (D1) of CD4 that overlaps with the binding site of Clenoliximab, whereas OKT4 binds to a nonoverlapping epitope within D3D4. Chronic treatment with Clenoliximab did not result in a decrease in OKT4 or CD8 binding below the range in naive animals, indicating it did not deplete these cells. OKT4A binding was inhibited initially following the repeated administration of Clenoliximab at the time of vector 1. OKT4A binding returned to normal levels during periods when there was no treatment with Clenoliximab for 7 days. The OKT4A epitope was masked again when blood samples were drawn from the mice within a few days of Clenoliximab dosing. These data are consistent with other observations, which show the presence of circulating Ab for 3–7 days at the 2-mg dose used in this study (data not shown).

PBMC were harvested at the time of administration of vectors 2 (day 28), 3 (day 60), and 4 (day 90) in animals chronically administered Clenoliximab and were analyzed for proliferation to adenovirus Ags (Fig. 6A) and SEB (Fig. 6B). Serum (Fig. 6C) and BAL (Fig. 6D) were also harvested for neutralizing Ab to adenovirus. Fig. 7 shows the outcome of gene transfer.
Chronic treatment with Clenoliximab resulted in persistent suppression of Ag-specific CD4^+ T cell responses to adenovirus Ags despite multiple challenges with vector (Fig. 6A). Similarly, stimulation to SEB was persistently diminished (Fig. 6B). Neutralizing Ab in serum and BAL were also suppressed (Fig. 6C and D). As predicted, this allowed for efficient administration of vector 2 (ALP, Fig. 7G), vector 3 (GFP, Fig. 7H), and vector 4 (luciferase), which did not occur when animals were not treated with Clenoliximab (2) (Fig. 7, A–E).

Study group 6 was designed to evaluate the effect of Clenoliximab after the activity of the Ab had waned. Vector 1 was administered with a short course (spanning days 23 to 128) of Clenoliximab, and vector 2 was administered on day 90. Binding of OKT4A to PBMC returned to normal by day 43 (Fig. 5A), as did the responsiveness of these cells to SEB by day 60 (Fig. 6B). Importantly, Ag-specific responses to adenovirus (Fig. 6A) and neutralizing Ab in serum (Fig. 6C) and BAL (Fig. 6D) all remained suppressed beyond the time Clenoliximab was no longer active. Ad-GFP was effectively readministered on day 90 (Fig. 7J).

Transient inhibition of CD4^+ T cells also markedly prolonged the expression of lacZ from the first vector administration, consistent with its suppression of CTL activity (Fig. 8).

**Discussion**

In this study we investigated the ability of a nondepleting mAb to human CD4 to eliminate humoral and cellular immune responses to adenovirus. A unique murine model was used for analyzing the impact of inhibition of human CD4 on adenoviral vector-mediated gene transfer to lung. This mouse is genetically deficient in murine CD4 by virtue of a gene disruption and is transgenic for human CD4 under the control of a T cell-specific promoter. Using this animal model we have demonstrated the efficacy of a nondepleting anti-human CD4 Ab to inhibit generation of T and B cell immune responses, which allow repeated administration of adenoviral vectors in the lung.

The critical role of CD4 molecules in regulation of T cell functional responses is clearly established. CD4-MHC class II interactions have been shown to be involved in both enhanced cell adhesion and transducing signals to T cells, which contribute to the strength of the TCR/CD3-mediated signal (15, 22, 23). The nature of the signals transduced through the CD4 molecule that contribute to TCR/CD3 signals have been extensively studied (24). The in vivo role of CD4 molecules in differentiation of Th cells was elucidated in early studies in muCD4KO mice, which suggested that the CD4 molecule was not required for some Th1 cell functions.

**FIGURE 6.** Cellular and humoral immune functions in HuCD4 mice treated with Clenoliximab (CD4 mAb). This figure summarizes the T cell proliferative and humoral responses in HuCD4 mice treated with multiple doses of vector in the presence or the absence of Clenoliximab. The following conditions were evaluated: naive animals (no vector; lightly shaded bars); treatment with vector alone and analyzed on day 28 (vector alone; open bars); group 6, treatment of vector on days 1 and 90 with a short course of Clenoliximab at the time of vector 1 (vector + CD4 mAb, short course; filled bars); and group 5, animals received four sequential doses of vector with chronic treatment with Clenoliximab (vector + CD4 mAb, chronic treatment; heavily shaded bars). A and B, Splenocytes obtained on various days were analyzed for adenovirus- and SEB-induced lymphoproliferative responses as described in Materials and Methods. Data are presented as the stimulation index, which is the incorporation of radioactivity in the presence of stimulator (i.e., adenovirus or SEB) over the incorporation of isotope when cells are incubated with medium alone. Analyses were performed on days 28, 60, and 90. A, Adenovirus-induced lymphoproliferative (LPR) responses; B, SEB-induced LPR responses. C and D, The humoral immune responses of animals that received multiple administrations of vector in the presence or the absence of Clenoliximab. C, Neutralizing Ab from serum; D, neutralizing Ab from BAL (both presented as reciprocal dilutions). Samples were analyzed on days 28, 60, 71, and 90. Animals treated with vector without Clenoliximab were analyzed on day 28 (vector alone). A full spectrum of neutralizing Ab analyses were performed from groups 5 (vector + CD4 mAb, chronic treatment) and 6 (vector + CD4 mAb, short course). ND, not done.
Two recent studies using *Nippostrongylus brasiliensis* and *Leishmania major* have demonstrated that the CD4 molecule is critical for Th2 cell differentiation. In our study, administration of Ad-*lacZ* to muCD4KO mice generated a detectable, but significantly decreased, Th1-type response with the absence of a Th2-type response. Reconstitution of immune function through the human CD4 transgene in the HuCD4 mouse resulted in humoral and cellular immune responses to vector that were equivalent to those observed in MHC-matched BALB/c mice (data not shown). Similar effects have been reported in clinical trials with anti-CD4 Abs in humans (27). These observations are consistent with the finding that CD4 molecules contribute to differentiation of the immune response to a Th2 phenotype (22, 26). Thus, HuCD4 mice generate normal immune responses and, therefore, are a good model for evaluating therapeutics that interfere with human CD4 function.

Previous studies using depleting Abs to murine CD4 in adenovirus vector-treated immune (28)-competent mice have demonstrated that the helper functions of CD4<sup>+</sup> T cells are critical for induction of CD8<sup>+</sup> CTL and secretion of neutralizing Ab by B cells. Clenoliximab is being developed for the treatment of rheumatoid arthritis, an autoimmune disease, and it is a potential candidate as an adjunct to in vivo gene therapy in humans with viral infections.
vectors. Administration of Clenoliximab caused coating of but did not lead to depletion of CD4 T cells. The change in CD4 and CD8 T cells is a dynamic process, since adenovirus vector instillation resulted in expansion of T cells, which was affected by Clenoliximab treatment. T cell responsiveness was markedly inhibited in Clenoliximab-treated animals, as shown by loss of both adenovirus and SEB responses on days 11–28. Interestingly both short course and chronic regimens of Clenoliximab resulted in persistent loss of Ag-specific responsiveness following an exposure to vector. The restoration of SEB responses following a short course of Clenoliximab correlated directly with the recovery of the OKT4A epitope in these mice, which corresponds to the loss from circulation of any remaining Clenoliximab. The prolonged suppression of Ag-specific immune responses (including generation of neutralizing Abs) in animals receiving a short course of Clenoliximab was confirmed by the ability to readminister the Ad-GFP on day 90 (see group 6). These results demonstrate that a 4-wk regimen of CD4 Clenoliximab leads to long term abrogation of Ag-specific immune responses to the coadministered vector. This appears to be due to the complete suppression of the initial response, allowing the mice to behave as in first time exposure to Ag and is not tolerance because a secondary exposure to vector without co-administration of Clenoliximab will stimulate normal responses to vector (data not shown).

We next asked whether adenoviral vectors could be repeatedly administered under conditions of CD4 T cell suppression, which would better simulate clinical applications. An experimental protocol was developed in which virus expressing different transgenes was instilled into the lung at 4-wk intervals along with i.p. injections of Clenoliximab. This study demonstrates that Clenoliximab is very effective in inhibiting the generation of neutralizing Ab responses after multiple injections of vector. The lack of functional humoral responses was confirmed by the ability to readminister adenoviral vectors with four sequential treatments. These findings indicate that Clenoliximab is an effective immunosuppressive agent to block generation of humoral and cellular immune responses during repeated adenovirus vector administrations to the lung.

In conclusion, the ability to readminister vectors will be critical for the successful application of gene therapy for chronic diseases.
Inhibition of CD4+ T cell function with a nondepleting Primatized anti-human CD4 Ab effectively blocks the T cell-dependent B cell response to adenoviral vectors in the lung. Application of this strategy for gene therapy to lung, such as CF, will require a more careful assessment of safety.

Acknowledgments

The scientific collaboration of the Vector, Cell Morphology, and Immunology (Ruth Qian, George Qian, and Parag Dhagat) Cores of the Institute for Human Gene Therapy is greatly appreciated. Marcia Houston-Leslie and Rosalind Barr provided excellent technical assistance. We acknowledge the Clenoliximab Project Team at IDEC Pharmaceuticals and Smith-Kline Pharmaceuticals for advice and support.

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