Phase I Trial of Recombinant Adenovirus Gene Transfer in Lung Cancer

Longitudinal Study of the Immune Responses to Transgene and Viral Products

Hanne Gahéry-Ségard,* Valérie Molinier-Frenkel,‡ Christophe Le Boulaire,‡ Patrick Saulnier,‡ Paule Opolon,‡ Renée Lengagne,* Eric Gautier,‡ Axel Le Cesne,‡ Laurence Zitvogel,‡ Alain Venet,* Christian Schatz,§ Michael Courtney,§ Thierry Le Chevalier,‡ Thomas Tursz,‡ Jean-Gérard Guillet,* and Françoise Farace‡

**Laboratoire d'Immunologie des Pathologies Infectieuses et Tumorales, INSERM Unité 445, Institut Cochin de Génétique Moléculaire, Université R. Descartes, Hôpital Cochin, 75014 Paris, France; ‡Institut Gustave Roussy, Départements de Biologie Clinique ou de Médecine, 94805 Villejuif, France; and §Transgène, 67082 Strasbourg, France*

Abstract

Animal studies indicate that the use of replication-deficient adenovirus for human gene therapy is limited by host anti-vector immune responses that result in transient recombinant protein expression and blocking of gene transfer when rechallenged. Therefore, we have examined immune responses to an adenoviral vector and to the β-galactosidase protein in four patients with lung cancer given a single intratumor injection of 10^9 plaque-forming units of recombinant adenovirus. The β-galactosidase protein was expressed in day-8 tumor biopsies from all patients at variable levels. Recombinant virus DNA was detected by PCR in day-30 and day-60 tumor biopsies from all patients except patient 1. A high level of neutralizing antiadenovirus antibodies was detected in patient 1 before Ad-β-gal injection whereas it was low (patient 3) or undetectable in the other two patients. All patients developed potent CD4 type 1 helper T cell (Th1) responses to adenoviral particles which increased gradually over time after injection. Antiadenovirus cytotoxic T lymphocyte responses were consistently boosted in the two patients examined (patients 3 and 4). Sustained production of anti–β-galactosidase IgG was observed in all patients except patient 1. Consistent with anti–β-gal antibody production, all patients except patient 1 developed intense, dose-dependent Th1 responses to soluble β-galactosidase which increased over time. Strong β-galactosidase–specific cytotoxic T lymphocyte responses were detected in patients 2, 3, and 4. Our results clearly show that despite the intensity of antiadenovirus responses, transgene protein expression was sufficient to induce strong and prolonged immunity in three patients. Recombinant adenovirus injected directly into the tumor is a highly efficient vector for immunizing patients against the transgene protein. (*J. Clin. Invest.* 1997. 100:2218–2226.) Key words: gene therapy • humans • recombinant adenovirus • cancer • immune responses

Introduction

Replication-defective adenoviruses are attractive candidates for in vivo gene transfer because of their ability to transduce a wide variety of cell types (1, 2). The first generation vectors, rendered replication defective by the deletion of the E1 sequences, efficiently transfer genes to nonproliferating cells and thus offer great advantages to cancer therapy because of the heterogeneity of tumor cell cycling (3–6). Moreover, the transient nature of virus expression and the fact that viral DNA is not integrated into the host cell genome avoid potential risks of insertional mutagenesis and allow recombinant protein expression in tumor cells without long-term persistence of the vector in nonmalignant cells. Therefore, several adenoviral recombinant vectors have been engineered for cancer gene therapy. They allow either the transfer of tumor suppressor genes (7, 8), or toxic gene products that promote tumor cell death (9), or the transfer of genes encoding cytokines (10, 11) or tumor antigens (12–14) designed to stimulate host immune responses against malignant cells.

Studies in animal models indicate that host immune responses, including cytotoxic T lymphocytes (CTL)[1] specific for virus-infected cells, contribute to the transient expression of the transgene and are associated with the development of local inflammation with the infiltration of lymphocytes and polymorphonuclear leukocytes (15–17). Recombinant adenovirus vectors also induce humoral and cellular immunity directed to the recombinant protein (18) and a recent study indicates that this immune response may be responsible for the loss of transgene expression (19). The input viral proteins recognized by T helper lymphocytes (Th) lead to the formation of neutralizing antibodies that prevent gene transfer when a second injection of recombinant virus is administered (20, 21). However, the immunogenicity of adenoviral vectors that has emerged as a critical issue for therapy of genetic disease is not necessarily an obstacle to tumor gene therapy. The local inflammation induced by the vector may boost the immune system and stimulate preexisting antitumor CTL which may contribute to tumor regression. As immune responses may either limit gene transfer efficiency or contribute to the antitumor therapeutic benefit, careful studies in human settings are required.

Address correspondence to F. Farace, Unité d'Immunologie Cellulaire, Département de Biologie Clinique, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif, France. Phone: 33-1-42-11-44-78; FAX: 33-1-42-11-52-68.

Received for publication 11 April 1997 and accepted in revised form 29 August 1997.

J. Clin. Invest.

0021-9738/97/11/2218/09 $2.00
Volume 100, Number 9, November 1997, 2218–2226
http://www.jci.org

1. *Abbreviations used in this paper:* β-gal, β-galactosidase; Ad-β-gal, recombinant replication-defective adenovirus containing the *lac*Z gene; Ad-rec, recombinant replication-defective adenovirus; ALVAC-β-gal, recombinant canary pox virus containing the *lac*Z gene; CTL, cytotoxic T lymphocytes; CTLp, cytotoxic T lymphocyte precursors; LDA, limiting dilution analysis; PFU, plaque-forming unit; Th1, type 1 helper T cells; Vac, wild-type vaccinia virus; Vac-β-gal, recombinant vaccinia virus containing the *lac*Z gene.

Based on compelling preclinical data, a phase I study was conducted to evaluate the feasibility and tolerance of a single intratumor injection of a recombinant replication-deficient adenovirus containing the bacterial *lac*Z gene (Ad-β-gal), in patients with inoperable lung cancer (22). We investigated the humoral and cellular immune responses directed against the β-galactosidase (β-gal) protein and to the adenoviral vector in four patients administered with 10^9 plaque-forming units (PFU). Extensive analysis of the immune responses induced in humans by adenovirus-mediated gene therapy has not been reported so far. Strong immune responses directed against the vector and the β-gal protein were induced, clearly indicating that the immunogenicity of the adenoviral vector does not impede prolonged immune responses against the transgene product in humans. These results also support the view that a single intratumor injection of recombinant adenovirus is an efficient procedure for immunizing patients against the transgene protein.

Methods

Patients and study design. The clinical protocol and study design have been described previously (22). Briefly, a replication-defective recombinant adenovirus was constructed using a modified type 5 adenoviral genome. The Rous sarcoma virus promoter was used to drive the transcription of the *Escherichia coli* gene encoding for the enzyme β-gal (2). Infectious Ad-β-gal stocks were prepared by Transgène. Patients with locally advanced lung cancer and an endobronchial lesion accessible by fiberoptic bronchoscopy were considered eligible for the study. The patients had not received chemotherapy before Ad-β-gal injection, but a standard chemotherapy regimen was started 3 d after Ad-β-gal injection. The viral suspension was given on day 0 with a fiberoptic bronchoscope. Doses of 10^7, 10^8, and 10^9 PFU were given in a single injection to cohorts of three, three, and six patients, respectively. Bronchoscopies were performed on day 8 and 1, 2 and 3 mo after viral inoculation to assess the macroscopic antitumor response and to investigate the transgene expression on biopsies of the endobronchial tumor. The immunologic study reported here concerned the first four patients who received 10^9 PFU between January and July of 1996. Blood samples were collected for this study on day 0 (before treatment), day 8, and 1, 2, 3, and 4 mo after Ad-β-gal injection. PBMC and serum were isolated by standard methods and preserved frozen.

PCR for recombinant adenovirus DNA detection. The nested PCR procedure used to detect recombinant adenovirus genes has been reported previously (22). Briefly, specimens were lysed using virus lysis buffer (10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20) and 0.2 mg/ml proteinase K. After incubation at 56°C for 1 h and further incubation at 100°C for 10 min, DNA was extracted with an equal volume of phenol/chloroform isoamyl-alcohol. Positive control of extraction was performed using 250 PFU Ad-β-gal added to 10^6 293 cells (0.2 ml). Negative control of extraction was performed using 0.2 ml PBS. Two pairs of primers were used for the nested PCR as follows: pair No. 1, primers OTG5347 (lacZ gene nucleotides 4284–4307; GenBank ECOLAC) and OTG4908 (Ad5 nucleotides 3352–3329; GenBank AD5001) for the outer PCR, pair No. 2, primers OTG1 (lacZ gene nucleotides 4319–4340; GenBank ECOLAC) and OTG5349 (simian virus 40 polyadenylation nucleotides 2608–2631, GenBank SV40CG) for the inner PCR. PCR procedures were performed with 1× PCR buffer II (Perkin-Elmer Cetus, Norwalk, CT) and 2.25 mM $MgCl_2$. The outer PCR was performed in a 25 μl vol using 20 μM for each deoxynucleotide, 100 nM for each primer, 5 μl of DNA template, and 1.75 U of AmpliTaq polymerase (Perkin-Elmer Cetus). The reaction volume was overlaid with 50 μl of siliconed oil (NMI-390; Chemiewerk Nünchritz GmbH, Nünchritz, Germany), and 75 μl of inner PCR mix. The conditions for inner PCR were 60 μM for each deoxynucleotide and 200 nM for each primer. Cycling was performed in a 9600 thermocycler (Perkin-Elmer Cetus) as previously described (22). The tubes were centrifuged for 300 *g* for 10 min between the two 25-cycle PCR. Two PCR tubes were always performed in parallel for each biopsy specimen. DNA from the biopsy specimen was tested alone in the first tube and 2 fg of recombinant adenovirus DNA, as internal positive control, was added to the biopsy DNA in the second tube.

Cell lines and viruses for in vitro assays. The 293 cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Infected cells were harvested after 48 h and suspended in PBS at 10^7 cells/ml. 293 cells were disrupted by three freeze-thaw cycles and the supernatants were harvested. Each viral preparation was titrated by limiting dilution plaque assay on 293 cells. Other cell lines were cultured using RPMI medium (GIBCO BRL) supplemented as described above. For patients 2, 3, and 4, lymphoblastoid cell lines were established by culturing 5×10^6 PBMC with 1 ml supernatant containing infectious EBV particles in the presence of 0.1 μg/ml cyclosporin A for 1 mo. Ad-β-gal (provided by Transgène) was used to analyze the antiadenovirus CTL responses. A recombinant replication-defective adenovirus (Ad-rec) was used as a control. Ad-β-gal and Ad-rec were prepared from infected 293 cells. The anti–β-gal studies used a recombinant canary pox virus vector containing the *lac*Z gene (ALVAC-β-gal) provided by J. Tartaglia (Virogenetics, New York), a vaccinia-*lac*Z construct (Vac-β-gal) provided by R. Drillien (CNTS, Strasbourg, France), and a control vaccinia vector (Vac) provided by Transgène.

Adenovirus neutralization assay. Permissive 293 cells were distributed in 96-well plates at 20,000 cells/well and cultured in complete DMEM medium for 24 h. Ad-β-gal (0.3 PFU/cell) was incubated with serial dilutions of decomplemented serum for 1 h at room temperature. This mixture (100 μl) was incubated with the 293 cells for 1 h at 37°C. Complete DMEM medium (100 μl) was then added and the cells were cultured for 20 h. Cells were washed twice in PBS and incubated with 100 μl lysis buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 10 mM KCl, 0.1 mM $MgSO_4$, 50 mM β-mercaptoethanol, and 0.5% Triton X-100) containing 17 μg of 4-methyl-umbelliferyl β-D-galactoside (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C. The resulting fluorescence was measured at 360/460 nm with a Cytofluor 2300 (Millipore Corp., Bedford, MA). Serum containing neutralizing antibodies inhibited adenoviral infection, as shown by the absence of β-gal activity in 293 lysates which corresponded to 100% of neutralization.

Antiadenovirus helper T cell responses. Triplicate cultures of PBMC (10^5/well) were incubated with different concentrations of Ad-β-gal and Ad-rec (50–200 PFU/cell). Proliferation was generally determined on days 5, 6, and 7 in culture by adding 1 μCi/well of [^{3}H]thymidine (NEN, Paris, France) 18 h before harvesting. Results were expressed in counts per minute (Δ cpm) calculated as follows: $\Delta\ cpm = mean\ cpm\ adenoviruses - mean\ cpm\ mock$. The capacity of PBMC to proliferate in vitro was checked in independent cultures performed for 3 d with 1 μg/ml PHA. Triplicate cultures were set up for assay of IL-2, IL-4, and IFN-γ which were performed using Genzyme Corp. (Cambridge, MA), Cayman Chemical Co., Inc. (Ann Arbor, MI), and Immunotech (Marseille, France) ELISA kits, respectively.

Antiadenovirus CTL responses. PBMC (4×10^6) were stimulated once with 200 PFU/cell Ad-β-gal in 1 ml complete RPMI culture medium for 7 d. Cytotoxic activity was tested against the autologous EBV cell line infected for 2 h with mock or Ad-β-gal or Ad-rec. The infected EBV cells were washed and incubated overnight at 37°C in complete RPMI medium before ^{51}Cr (NEN) labeling. Cytolytic activity was determined in a standard 4-h ^{51}Cr-release assay. Results of duplicate cultures are expressed as: $\%\ specific\ Cr\ release = 100 \times (experimental - spontaneous\ release)/(total\ Cr\ incorporated - spontaneous\ release)$. The average spontaneous release never exceeded 20% of the total incorporated ^{51}Cr.

Anti–β-gal antibodies detected by ELISA. Soluble β-gal protein

Table I. Clinical Status and Expression of β-gal in Tumor Biopsies

Patient	Clinical status*			Expression of β-gal‡	
	Endoscopic response	Clinical response	Duration	Number of positive biopsies	% positive tumor cells§
			mo		
1	PR	PR	4	1/8	5–10%
2	MR	PR	3	3/9	5–10%
3	PR	PR	3	2/5	5–10%
4	SD	PD	—	5/6	45–55%

*The extent and duration of tumor regression were determined clinically and endoscopically for each patient. *PR*, Partial response, i.e., 50% or greater reduction in the sum of the products of the largest perpendicular diameters of measurable lesions; *MR*, minor response, i.e., > 25% decrease but < 50%; *SD*, stable disease, i.e., decrease of < 50% or an increase of < 25% with no new lesions; *PD*, progressive disease, i.e., increase > 25% or appearance of new lesions. ‡Expression of β-gal was determined in tumor biopsies obtained at day 8 after Ad-β-gal injection. Bronchial biopsies were embedded in OCT and preserved frozen in liquid nitrogen. Cryosections were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyronoside) as described (3). §Percentage of β-gal$^+$ tumor cells on a representative positive biopsy.

(Boehringer Mannheim, Mannheim, Germany) was used at 500 ng/well to coat 96-well plates (Maxisorb; Nunc, Inc., Naperville, IL). The blocking buffer (PBS, 0.1% Tween 20, 1% BSA) was added for 1 h. The plates were washed (PBS, 0.1% Tween 20) and dilutions of serum were incubated overnight at 4°C. The plates were washed and incubated with alkaline phosphatase–labeled sheep anti–human IgG or IgM (The Binding Site, Inc., San Diego, CA) for 2 h at room temperature. Enzymatic activity was detected using 4-methyl-umbelliferyl phosphate as substrate (Sigma Chemical Co.). The resulting fluorescence was measured with the Cytofluor 2300.

Anti–β-gal helper T cell responses. Triplicate cultures of PBMC (10^5/well) were performed in complete medium with various concentrations of soluble β-gal. Proliferation was measured by incorporating [^{3}H]thymidine after 5, 6, and 7 d of culture, as described above. Lymphokine assays were performed as described above.

Anti–β-gal CTL. In vitro stimulation of PBMC was performed by mixing 2×10^7 PBMC (responder cells) with 2×10^6 irradiated stimulating cells (autologous PBMC incubated for 2 h with 10^8 PFU ALVAC-β-gal) in complete RPMI culture medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes, nonessential amino acids, and 10% heat-inactivated FCS). On day 7, responder cells were restimulated by ALVAC-β-gal infected autologous PBMC (prepared as on day 0), in medium supplemented with 10 U/ml IL-2. On day 14, anti–β-gal CTL were tested by using the autologous EBV cell line infected overnight with Vac-β-gal or Vac (20 PFU/cell) as targets. For limiting dilution analysis (LDA), PBMC were seeded at eight concentrations (from 750 to 10^5 cells/well) and 24 replicates were set up for each concentration. PBMC were stimulated once with irradiated autologous PBMC infected by ALVAC-β-gal, as described above (10^4 cells/well). Cultures were fed with 10 U/ml IL-2 on days 3, 7, and 10, and cytolytic activity was tested against Vac-β-gal and Vac-infected targets on day 14. Cytolytic activity was determined in the ^{51}Cr-release assay as described above. For LDA, wells in which the ^{51}Cr released exceeded 10% (> 3 SD) were considered positive for cytolytic activity. The frequency of cytotoxic T lymphocyte precursors (CTLp) was estimated using the weighted mean method from the Poisson distribution relationship between the number of responding cells and the logarithm of the percentage of nonresponding cultures, as described by Taswell (23). The CTLp frequencies of the PBMC samples were compared using the χ^2 test (24).

Results

Expression of β-gal and persistence of recombinant virus DNA in tumor specimens. 12 patients with inoperable lung cancer were included in the phase I recombinant adenovirus-mediated gene transfer study. The feasibility and tolerance in the

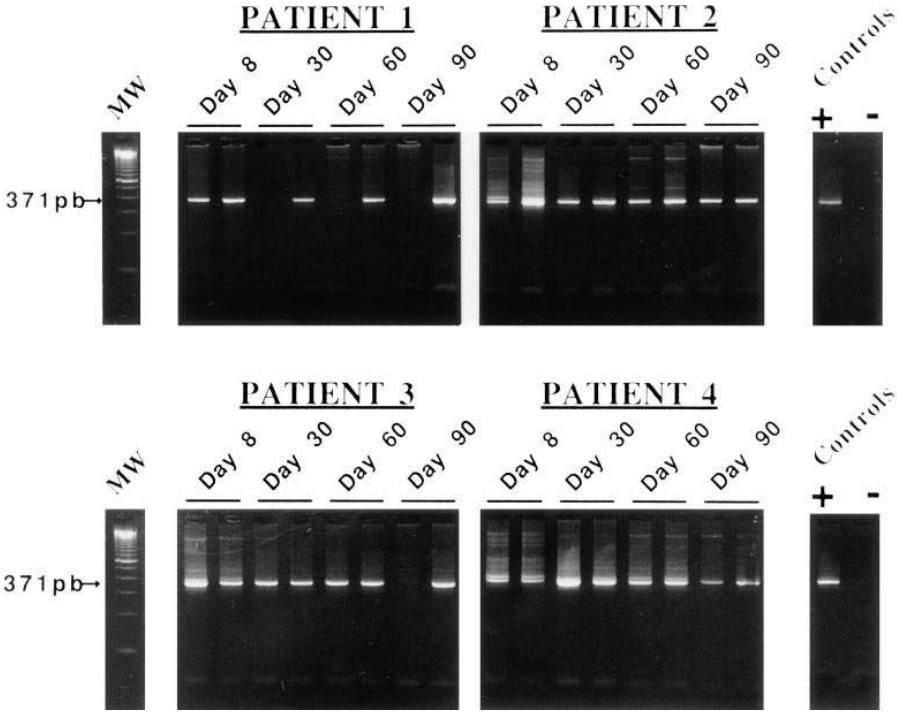

Figure 1. Persistence of recombinant virus DNA in tumor biopsy specimens. The presence of recombinant virus DNA in tumor biopsy extracts was monitored using a nested PCR procedure. The amplification products were stained by ethidium bromide and visualized on agarose gels. Experiments with day-8, day-30, day-60, and day-90 biopsies are shown for the four patients. For each day (8, 30, 60, 90), the first lane corresponds to results obtained with the tumor DNA extract and the second lane to the PCR internal positive control (i.e., amplification of 2 fg of a positive DNA control added to the tumor extract). Positive and negative controls of DNA extraction performed, as described in Methods, are shown for each series of experiment. Molecular weights were determined using 100-bp DNA ladder (Life Technologies, Inc., Gaithersburg, MD).

first six patients who received 10^7 and 10^8 PFU Ad-β-gal (three patients per dose level) have already been reported in detail (22). The present work is an analysis of immune responses in the first four patients given 10^9 PFU Ad-β-gal. All four patients had metastatic non–small cell lung carcinoma. A combination of vinorelbine and cisplatin was administered 3 d after the intratumor injection of Ad-β-gal. Two out of the four patients achieved an endoscopic objective response (Table I).

β-gal expression and the persistence of recombinant virus DNA were determined in tumor biopsy specimens obtained on day 8 and 1, 2, and 3 mo after virus inoculation. β-gal activity was detected on day-8 cryosections from all four patients (Table I). However, the level of β-gal expression varied considerably since a single biopsy was positive in patient 1 (out of eight) while three, two, and five positive biopsies were detected in patient 2, 3, and 4, respectively. The percentage of β-gal expression in positive biopsies was also variable (Table I). β-gal activity was not detectable in subsequent biopsies excepted in 1- and 2-mo specimens from patient 3 (data not shown). Recombinant viral DNA was detected by PCR in day-8 biopsies from all patients (Fig. 1). However, it was not detected in subsequent biopsies of patient 1 whereas it persisted for up to 2 mo in patient 3 and up to 3 mo in patients 3 and 4 (Fig. 1).

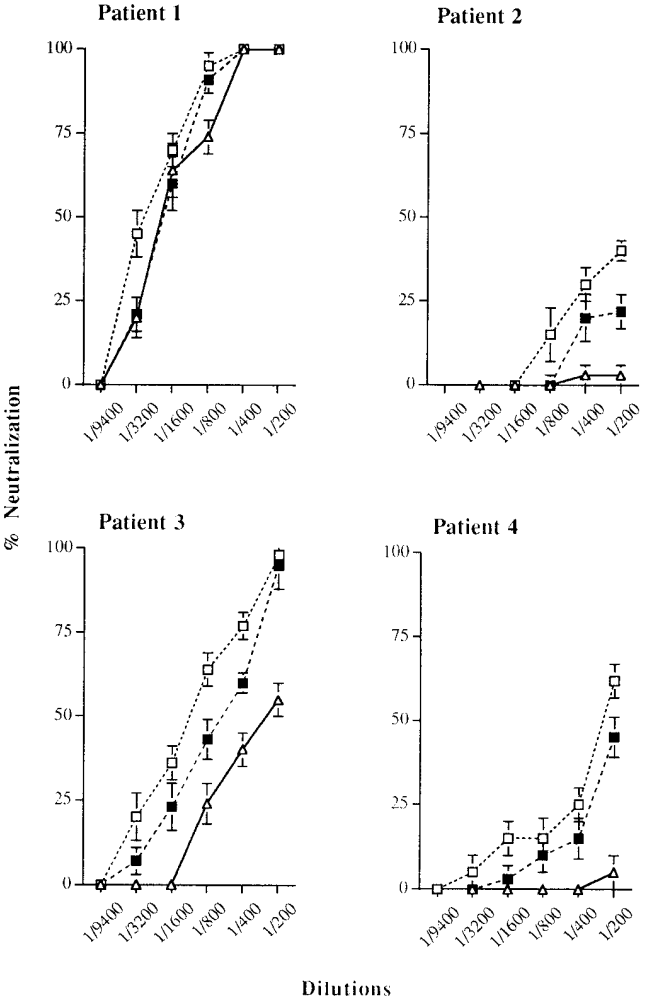

Figure 2. Neutralizing antiadenovirus antibodies. Various dilutions of serum collected on days 0 (*open triangles*), 30 (*open squares*), and 60 (*filled squares*) were mixed with Ad-β-gal particles and the resulting suspension was used to infect permissive 293 cells. Adenoviral infection was inhibited by serum samples containing neutralizing antibodies, as shown by the absence of β-gal activity in 293 lysates (100% neutralization). Results are expressed as percent neutralization and are the means (±SD) of duplicate tests from two experiments.

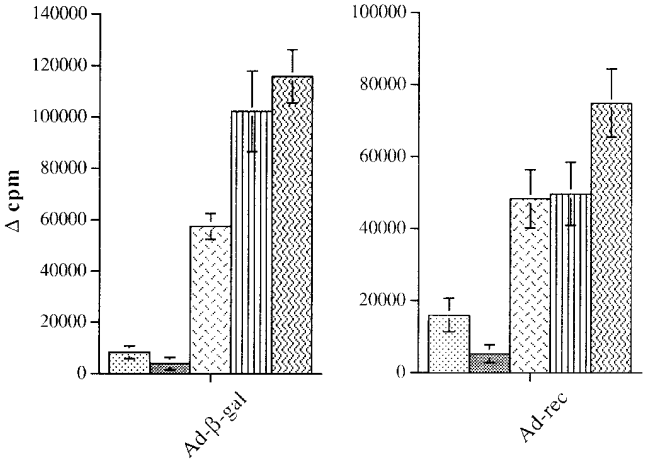

Figure 3. In vivo kinetics of PBMC proliferative response to recombinant adenovirus. PBMC collected from patient 3 on days 0 (*dotted bars*), 8 (*checkered bars*), 30 (*hatched bars*), 56 (*striped bars*), and 102 (*wavy striped bars*) after Ad-β-gal injection were cultured with Ad-β-gal and Ad-rec. Proliferation was measured by [^{3}H]thymidine incorporated on day 6. Means (±SD) of triplicate cultures from two experiments were expressed in counts per minute (Δ cpm) as indicated in Methods. All samples proliferated in response to 1 μg/ml PHA.

Neutralizing antiadenovirus antibodies. Pretreatment neutralizing antibodies reflect preexisting immunity to adenovirus. Diluted serum samples taken from the four patients on days 0, 30, and 60 were tested for antiadenovirus antibodies using a neutralization assay. Patient 1 had a high level of neutralizing antibodies before treatment (100% neutralization at 1/400) which remained stable after Ad-β-gal injection (Fig. 2). Patient 3 had a low titer of neutralizing antibodies (30% neutralization at 1/400) on day 0 which increased very significantly by day 30 and remained stable on day 60 (Fig. 2). Patients 2 and 4 had no detectable neutralizing antibodies on day 0. A low level of neutralizing antibodies was induced on day 30 but it decreased after 2 mo in these two patients (Fig. 2).

Helper T cell responses to recombinant adenoviruses. Dose-dependent proliferative responses to Ad-β-gal and Ad-rec particles were observed in all patients (data not shown). Experiments using pre- and postinjection PBMC were performed in order to assess the in vivo kinetics of response to viral antigens. As shown in a representative experiment performed in patient 3 (Fig. 3), weak proliferation was detected on days 8 and 30, which increased on days 56 and 102. Lymphokine (IL-2, IL-4, IFN-γ) secretion was also evaluated. IL-2 was secreted in response to viral antigens 3 d after the initiation of the culture and IFN-γ production was maximal on day 6. Representative results obtained with day-30 and day-102 PBMC (patient 3) are shown in Fig. 4. Both adenoviruses induced high levels of IL-2 and IFN-γ synthesis. In agreement with proliferation ex-

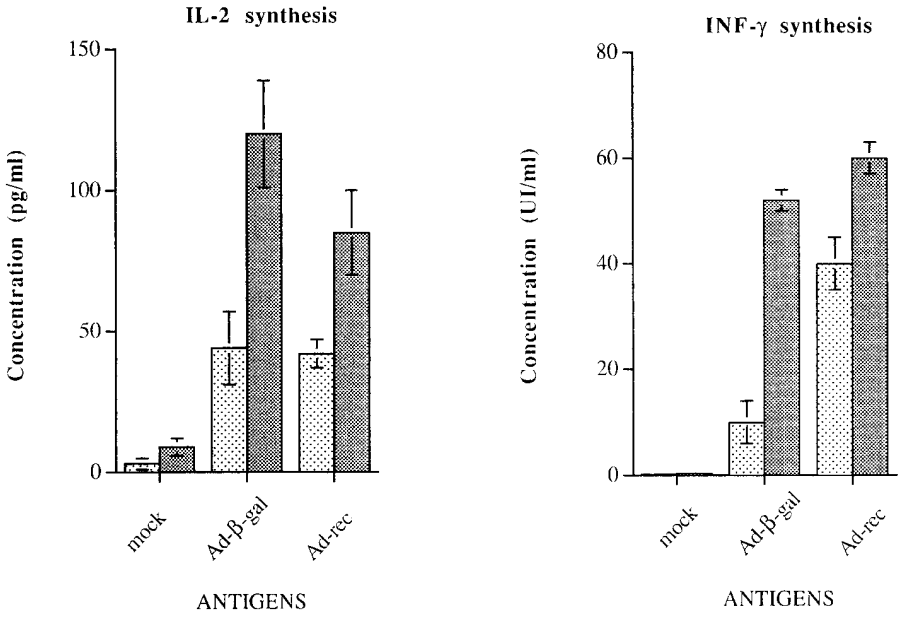

Figure 4. Secretion of Th1 cytokines in response to adenovirus antigens. PBMC collected from patient 3 on days 30 (*dotted bars*) and 102 (*checkered bars*) after Ad-β-gal injection were cultured with mock, Ad-β-gal, and Ad-rec. Supernatants of triplicate cultures were collected on day 3 for IL-2 determination and on day 6 for IFN-γ. Results were expressed as the means (±SD) of the three values.

periments, more IL-2 and IFN-γ was produced on day 102 than on day 30. No IL-4 was detected in culture supernatants (data not shown).

Cytotoxic T cell responses to recombinant adenovirus. As few blood samples were available in patients 1 and 2, antiadenovirus CTL responses were investigated only in patients 3 and 4. PBMC were stimulated once in vitro with Ad-β-gal particles for 7 d. The primed cells were then tested for their ability to lyse the autologous EBV cell line infected with different recombinant defective adenoviruses (Ad-β-gal, Ad-rec). As shown in a representative experiment (Fig. 5), cytotoxic activity specific for viral antigens, expressed by targets infected with the recombinant vectors, was detected in blood samples of patients 3 and 4 collected after Ad-β-gal injection. In contrast, no cytotoxic activity was detected before injection (Fig. 5). Thus, a single in vitro stimulation (7 d) resulted in significant CTL responses only for PBMC collected after treatment. These data suggest a major in vivo induction of antiadenovirus CTL responses by Ad-β-gal.

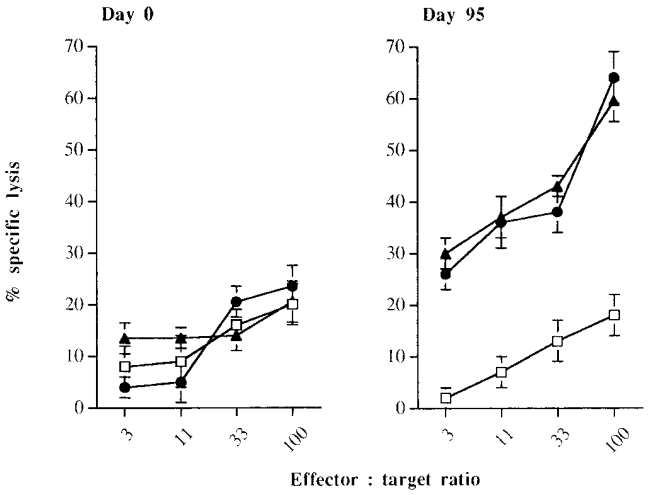

Figure 5. In vivo priming of adenovirus-specific CTL. PBMC from patient 4 collected on days 0 and 95 were stimulated in vitro once with Ad-β-gal particles for 7 d. Cytotoxic activity against the autologous EBV cells infected with mock (*open squares*) or Ad-β-gal (*filled circles*) or Ad-rec (*filled triangles*) was measured in a 4-h ^{51}Cr-release assay. Results are the means (±SD) of duplicate tests from three independent experiments.

Humoral response to β-gal. Serum samples collected on days 0, 30, and 60 were assayed for anti–β-gal antibodies by ELISA. No IgGs specific to the β-gal protein were detected before injection. Serum samples from patients 2, 3, and 4 had high anti–β-gal IgG on day 30, which persisted on day 60 (Fig. 6). Thus, a single intratumor injection of 10^9 PFU Ad-β-gal induced a strong humoral IgG response. No β-gal–specific IgGs were detected in patient 1 even 2 mo after Ad-β-gal injection (Fig. 6). Patients 2, 3, and 4 also had high titers of anti–β-gal IgM on day 30 (but not patient 1), confirming the induction of primary anti–β-gal immune response in these patients (data not shown).

Helper T cell responses to β-gal. The proliferative response to soluble β-gal was examined in the four patients. Proliferation was strong and was dependent on the dose of β-gal in all patients except patient 1. The results obtained with PBMC from one of the three responders and from the nonresponder are shown in Fig. 7 *A*. The kinetics of the in vivo response to β-gal were examined by assaying a pre- and postinjection series of PBMC in the same experiment. Anti–β-gal responses increased gradually from days 30 to 102 after Ad-β-gal injection, as shown in a representative experiment performed in patient 3 (Fig. 7 *B*). No proliferation was detectable in day-0 PBMC (Fig. 7 *B*). In agreement with these results, no anti–β-gal response was detected in normal subjects (n = 4) nor in patients (n = 2) with invasive *E. coli* infection (data not shown).

PBMC collected at different times after Ad-β injection were also tested for their capacity to produce IL-2, IFN-γ, and IL-4 in response to soluble β-gal. IL-2 and IFN-γ production

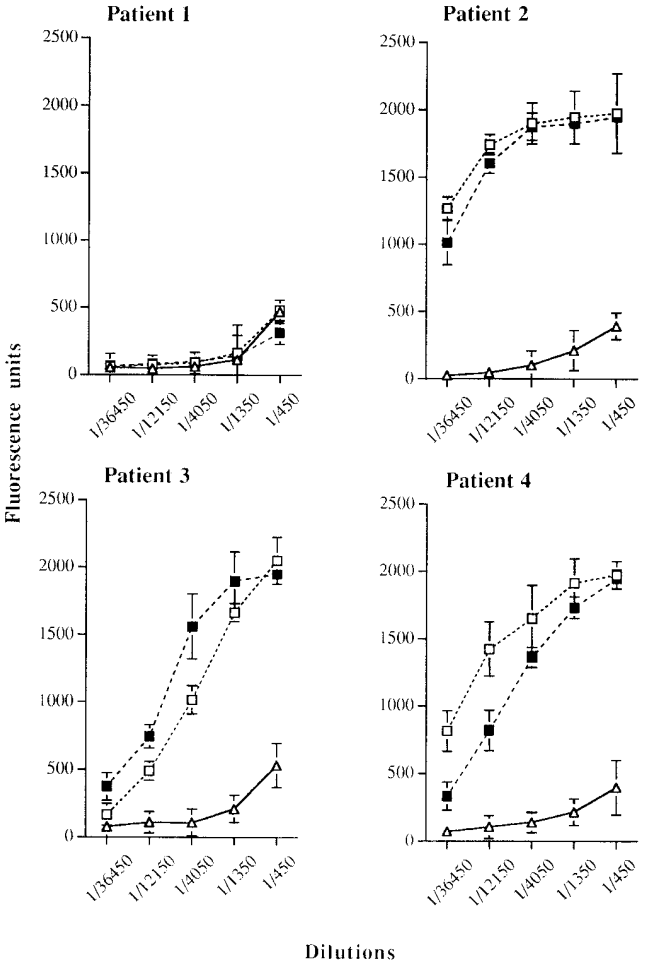

Figure 6. Anti–β-gal IgG antibodies. Dilutions of serum collected on days 0 (*open triangles*), 30 (*open squares*), and 60 (*filled squares*) were incubated with purified β-gal coated on microtiter plates. Anti–β-gal IgGs were detected using alkaline phosphatase–labeled sheep anti–human IgG antibodies and the enzymatic activity was detected using a fluorescent substrate. Results are the means (±SD) of duplicate tests from three experiments.

peaks were detected 2 and 4 d, respectively, after the initiation of cultures. High concentrations of IL-2 and IFN-γ were secreted according to the dose of β-gal, as shown in Table II. In contrast, no IL-4 was detected.

Cytotoxic T cell responses to β-gal. Anti–β-gal CTL responses were investigated in patients 2, 3, and 4. β-gal specific cytotoxic activity was tested against the autologous EBV cell line infected with either Vac-β-gal or Vac. Strong anti–β-gal responses were detected in PBMC collected after injection of Ad-β-gal for all three patients, as shown in Fig. 8 *A*. The anti–β-gal cytotoxic responses were further examined by cloning cultures displaying cytolytic activity (patient 3). HLA class I restriction was assessed by testing clones against Vac-β-gal–infected HLA class I–mismatched targets. No killing was detected, while the clones efficiently lysed Vac-β-gal–infected autologous targets (data not shown). The killing of Vac-β-gal–infected autologous EBV cells was also found to be blocked by an anti–class I monoclonal antibody (data not shown), confirming that the anti–β-gal cytolytic activity was mediated by CTL.

To monitor the in vivo induction of the β-gal–specific cytotoxic response over time for the same patient, we quantified CTLp by LDA. The same LDA experiment was used to determine the frequency of CTLp in PBMC collected from patient 3 on day 0 (before injection), and 2 and 4 mo after injection (Fig. 8 *B*). The frequency of β-gal–specific CTLp before injection (1/44652) was not different from that of the control vaccinia virus specific CTLp (1/41280). The LDA also indicated that the frequencies of β-gal–specific CTLp at 2 (1/10884) and 4 mo (1/12768) were equally elevated and different from that obtained on day 0. Consistent with day-0 LDA results, no anti–β-gal cytotoxic responses were detected in the PBMC from two healthy subjects (data not shown). In addition, LDA experiments performed in patient 2 also indicated elevated anti–β-gal CTLp frequencies at 2 mo after injection that were maintained at 4 mo (data not shown).

Discussion

Four lung cancer patients were enrolled in a phase I study of gene therapy and were given a single intratumor injection of 10^9 PFU recombinant adenovirus containing the *lac*Z marker gene. Tumor biopsy specimen collected on day 8, and 1, 2, and 3 mo after gene therapy were analyzed for β-gal activity and for the presence of recombinant viral DNA by PCR. β-gal activity was detected in day-8 biopsy specimen from all four patients but with notable variability. A single biopsy sample (out of nine) was positive in patient 1, suggesting that β-gal expression was lower in this patient than in the other three. In contrast, high β-gal expression was found in patient 4. Recombinant virus DNA persisted in patients 2, 3, and 4 for up to 2–3 mo, whereas only the day-8 biopsy sample was positive in patient 1. These results indicate that the β-gal marker gene had been successfully introduced into all patients but that the efficacy of gene transfer was variable.

We have examined cellular and humoral responses to the adenoviral vector and the β-gal protein in these patients. Neutralizing antibodies reflect the immunity to adenovirus. Patient 1, who had a high level of neutralizing antiadenovirus antibodies before Ad-β-gal injection, did not produce helper nor hu-

Table II. IL-2 and IFN-γ Synthesis in Response to Soluble β-gal Protein

β-gal	IL-2	IFN-γ
μg/ml	*pg/ml*	*IU/ml*
0	0	0
0.5	24 (±0.8)	1.7 (±1.1)
1.5	64.5 (±15.1)	10.5 (±3.5)
5	122.3 (±9.7)	19.03 (±0.5)
PHA (1 μg/ml)	1360 (±46)	39.2 (±1.1)

PBMC were collected in patient 4 at day 56 after Ad-β-gal injection. Cells were cultured with increasing doses of β-gal. Supernatants were harvested at day 2 for IL-2 and at day 4 for IFN-γ dosage. IL-2 and IFN-γ concentrations were determined using Genzyme and Immunotech kits, respectively. Three experiments were performed in duplicate and results are expressed as the mean (±SD) from two experiments.

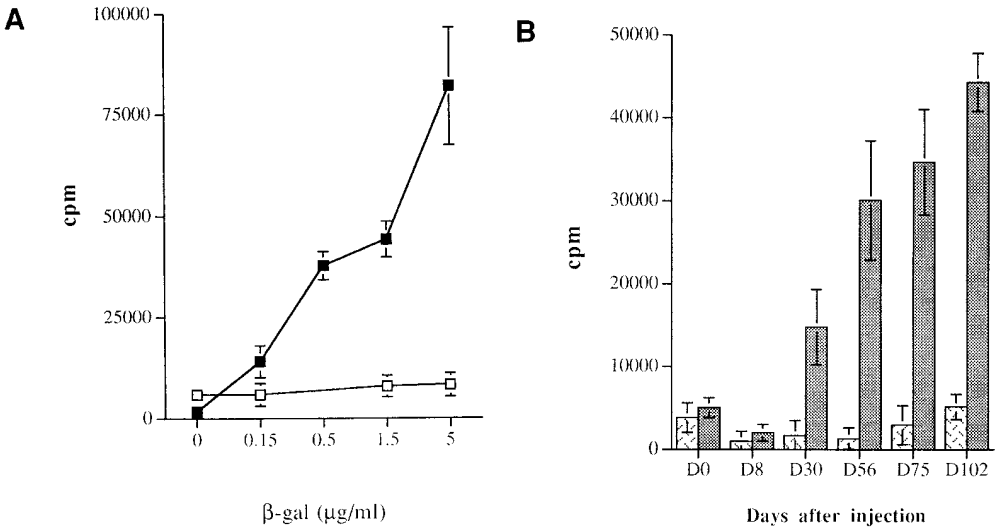

Figure 7. Proliferative responses of PBMC to soluble β-gal protein. (*A*) PBMC taken on day 84 from patient 1 (*open squares*) and on day 56 from patient 3 (*filled squares*) were cultured with increasing doses of β-gal for 6 d. Proliferation was measured by incorporating [^{3}H]thymidine and the means (±SD) of triplicate cultures from two experiments are shown. (*B*) The in vivo kinetics of the proliferative response of PBMC to soluble β-gal protein. PBMC from patient 3 on days 0, 8, 30, 56, 75, and 102 were cultured in medium alone (*hatched bars*) or with 1 μg/ml soluble β-gal (*checkered bars*) for 6 d. Proliferation was measured by incorporating [^{3}H]thymidine and the means (±SD) of triplicate cultures from two experiments are shown. All PBMC samples proliferated in response to 1 μg/ml PHA.

moral responses to the β-gal protein. Results in patient 1 suggest that the presence of a high level of neutralizing antibodies did not totally prevent viral penetration into cells but minimized the efficacy of gene transfer and is associated with little if any antitransgene immune responses. This is in agreement with mouse studies which show a direct correlation between the level of neutralizing antibodies and the blockage of gene transfer (20, 25). Patients had different preexisting immunity to adenovirus and responded differently to Ad-β-gal injection. Therefore, we postulate that the high level of neutralizing antibodies induced in patient 3 by Ad-β-gal injection will lower the efficacy of a second injection. In contrast, a moderate level of neutralizing antibodies will not prevent response to a second injection of recombinant adenovirus in patients 2 and 4.

We found that antiadenovirus CTL were strongly induced by Ad-β-gal injection, since a single in vitro stimulation (7 d in culture) produced a high level of specific cytotoxic activity. Recent studies have shown that in the majority of individuals, antiadenovirus CTL activities are detected using in vitro stimulation with dendritic cells or fibroblasts infected with adenovirus, after 2–3 wk in culture (26). Such activity seemed not predictive of high immunity to adenovirus which could be an obstacle to gene therapy.

Three patients developed strong primary immune responses to β-gal protein, as confirmed by the detection of anti–β-gal IgM antibodies. Potent helper T cell responses were associated with strong IgG production. In vitro stimulation of PBMC from these patients with viral antigens or β-gal protein resulted in the production of substantial quantities of IL-2 and IFN-γ, but no detectable IL-4. This indicates that the systemic immune responses elicited by an intratumor injection of Ad-β-gal are shifted towards a type 1 helper T cell (Th1) profile. Detailed analysis of locally produced cytokines will be needed since in mice, lymphocytes obtained by bronchoalveolar lavage also secreted IL-4, indicating that both Th1 and Th2 cells are activated in the airway (17). Injection of recombinant adenovirus containing the CFTR gene into the airway of patients with cystic fibrosis, induces a high level of IL-6 secretion and substantial inflammation (27).

The patients also developed strong CTL activity against the β-gal protein and the frequencies of CTLp in two patients taken 2 and 4 mo after the injection were similar, indicating that strong and prolonged immunity to the β-gal protein was induced despite the intensity of antiadenovirus responses. Overall humoral and cellular responses to β-gal protein reflect its immunogenicity, level of expression, and presentation to the immune system. The recombinant adenovirus used in this study contains the *lac*Z gene driven by a Rous sarcoma virus promoter which produces a high level expression of recombinant protein, in vivo. β-gal may be expressed by tumor cells and mononuclear infiltrates in the presence of both inflammatory and Th1 cytokines. This local environment may be adequate for priming anti–β-gal immune responses. The steady release of β-gal resulting from virus-specific cytolytic effector killing and/or the turnover of lung tumor cells may lead to efficient immunization. These patients were also given chemotherapy at the same time as gene therapy and this treatment did not prevent eliciting efficient immune responses directed against the transgene product. In the mouse, adenoviral vectors have been successfully used as vaccine vectors to immunize against a variety of viral pathogens (18, 28–32). This study clearly shows that a single intratumor injection of recombinant adenoviral vector allows for efficient immunization against the protein encoded by the transgene. These results support the clinical implementation of recombinant adenovirus coding for tumor antigens for cancer therapy.

Finally, strategies involving adenovirus-mediated transfer of tumor suppressor or toxic genes are based on the assumption that the transient expression of the recombinant protein is enough to promote lysis of the major tumor burden. Nonspecific inflammatory reactions and the cytokines released by antivirus effectors may also modify the tumor immunosuppressive environment and stimulate tumor-specific CTL, thereby boosting the therapeutic effect. Further studies are needed to

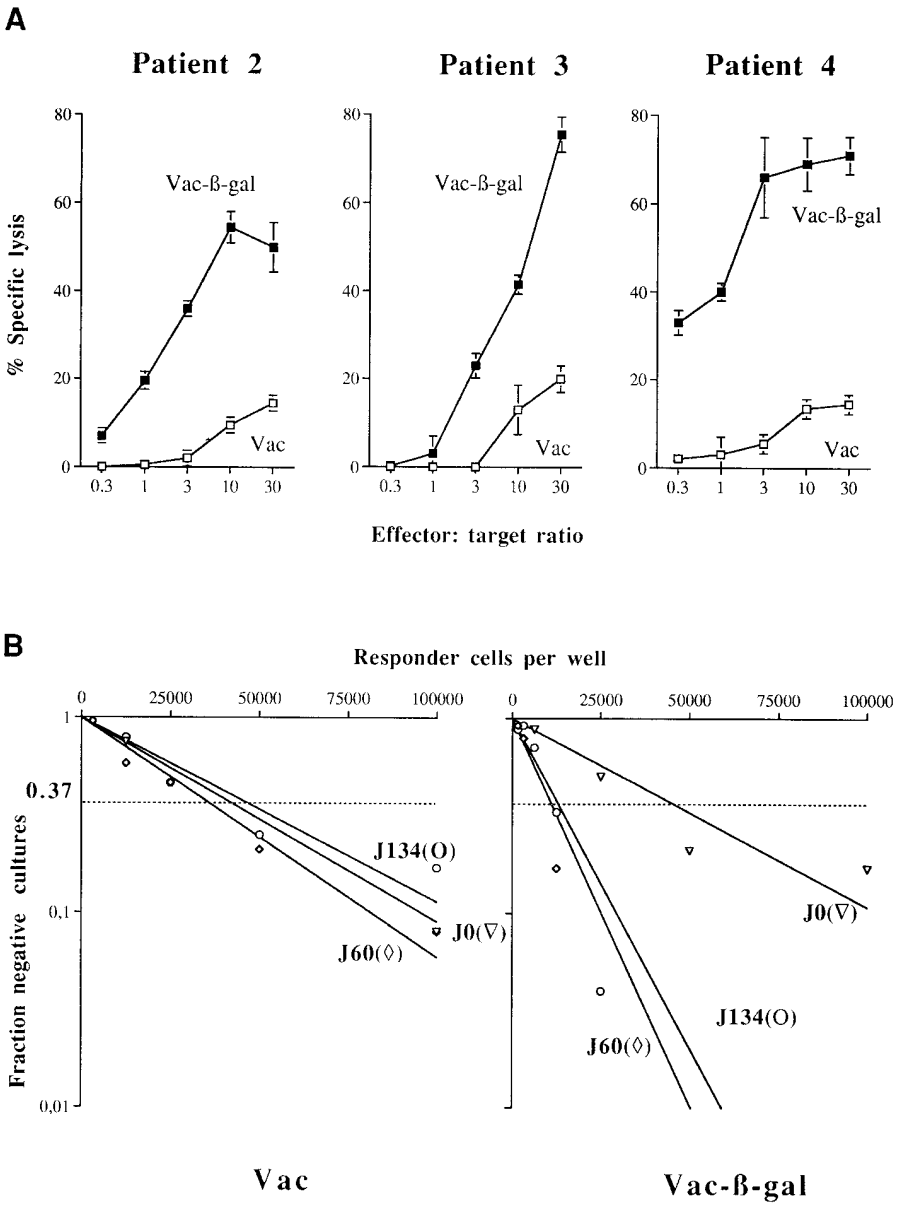

Figure 8. In vivo priming of β-gal–specific CTL. (*A*) Cytotoxic activity against the autologous EBV cell line infected with either the Vac-β-gal or the control Vac was measured in a 4-h ^{51}Cr-release assay. Results are expressed as the mean (±SD) of duplicate tests from three experiments. (*B*) The frequency of CTLp in patient 3 was estimated by LDA. PBMC collected before and 2 and 4 mo after Ad-β-gal injection were seeded at eight concentrations (from 750 to 10^5 cells/well). 24 replicates of each concentration were stimulated once with irradiated autologous PBMC infected by ALVAC-β-gal (10^4 cells/well). Replicates were tested against Vac-β-gal and Vac infected targets. Replicates in which ^{51}Cr release exceeded the mean spontaneous release by 10% were considered positive. CTLp frequency was estimated by the weighted mean method from the Poisson distribution relationship between the number of responding cells and the logarithm of the percentage of nonresponding cultures, as previously described (23).

determine whether the immune responses of cancer patients to the adenoviral vector limit or improve the therapeutic efficacy of these approaches.

Acknowledgments

The authors thank Dr. J. Tartaglia for providing the ALVAC-β-gal and Dr. R. Drillien for the Vac-β-gal. We also thank Dr. B. Escudier, Dr. M. Perricaudet, Dr. K. Benhioud, Dr. H. Haddada, Dr. C. Tancrede, Dr. S. Chouaib, and Dr. F. Faure for discussions and M. Dupuis for technical assistance.

This study was supported by grants from the Institut National de la Santé et la Recherche Médical (INSERM), from the Association pour la Recherche contre le Cancer (ARC), la Ligue Contre le Cancer, and the Association Française contre les myopathies (AFM). H. Gahéry-Ségard is supported by a fellowship from the ECS (Ensemble Contre le Sida, Sidaction, France).

References

1. Wilson, J.M. 1993. Vehicles for gene therapy. *Nature (Lond.).* 365:691–692.

2. Stratford-Perricaudet, L.D., M. Levrero, J.F. Chasse, M. Perricaudet, and P. Briand. 1990. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Hum. Gene Ther.* 1:241–256.

3. Stratford-Perricaudet, L.D., I. Makeh, M. Perricaudet, and P. Briand. 1990. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.* 90:626–630.

4. Quantin, B., L.D. Perricaudet, S. Tajbakhsh, and J.-L. Mandel. 1992. Adenovirus as an expression vector in muscle cells *in vivo*. *Proc. Natl. Acad. Sci. USA.* 89:2581–2584.

5. Zabner, J., L.A. Couture, R.J. Gregory, S.M. Graham, A.E. Smith, and M.J. Welsh. 1993. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *Cell.* 75:207–216.

6. Rosenfeld, M.A., K. Yoshimura, B.C. Trapnell, K. Yoneyama, E.R. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L.E. Stier, L. Stratford-Perricaudet, et al. 1992. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 68:143–155.

7. Clayman, G.L., A.K. El-Naggar, J.A. Roth, W.W. Zhang, H. Goepfert, D.L. Taylor, and T.J. Liu. 1995. In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. *Cancer Res.* 55:1–6.

8. Liu, T.J., A.K. El-Nagar, T.J. McDonnell, K.D. Steck, M. Wang, D.L. Taylor, and G.L. Clayman. 1995. Apoptosis induction mediated by wild-type p53 adenoviral gene transfer in squamous cell carcinoma of the head and neck. *Cancer Res.* 55:3117–3122.

9. O'Malley, B.W., J.S.H. Chen, M.R. Schwartz, and S.L.C. Woo. 1995. Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res.* 55:1080–1085.

10. Addison, C.L., T. Braciak, R. Ralston, W. Muller, J. Gauldie, and F.L. Graham. 1995. Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model. *Proc. Natl. Acad. Sci. USA*. 92:8522–8526.

11. Zhang, J.F., C. Hu, Y. Geng, J. Selm, S. Klein, A. Orazi, and M.W. Taylor. 1996. Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy. *Proc. Natl. Acad. Sci. USA*. 93:4513–4518.

12. Chen, P.W., M. Wang, V. Bronte, Y. Zhai, S.A. Rosenberg, and N.P. Restifo. 1996. Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. *J. Immunol.* 156:224–231.

13. Warnier, G., T. Duffour, C. Uyttenhove, T.F. Gajewski, C. Lurquin, H. Haddada, M. Perricaudet, and T. Boon. 1996. Induction of a cytolytic T-cell response in mice with a recombinant adenovirus coding for tumor antigen P815A. *Int. J. Cancer.* 67:303–310.

14. Zhai, Y., J.C. Yang, Y. Kawakami, P. Spiess, S.C. Wadsworth, L.M. Cardoza, L.A. Couture, A.E. Smith, and S.A. Rosenberg. 1966. Antigen-specific tumor vaccines. Development and characterization of recombinant adenoviruses encoding MART1 or gp100 for cancer therapy. *J. Immunol.* 156:700–710.

15. Yang, Y., C.J.E. Hildegund, and J.M. Wilson. 1994. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity.* 1:433–442.

16. Yang, Y., F.A. Numes, K. Berencsi, E.E. Furth, E. Gönczöl, and J.M. Wilson. 1994. Cellular immunity to viral antigens limits E1 deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA*. 91:4407–4411.

17. Yang, Y., Q. Li, H.C.J. Ertl, and J.M. Wilson. 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* 69:2004–2015.

18. Juillard, V., P. Villefroy, D. Godfrin, A. Pavirani, A. Venet, and J.-G. Guillet. 1995. Long-term humoral and cellular immunity induced by a single immunization with replication-defective adenovirus recombinant vector. *Eur. J. Immunol.* 25:3467–3473.

19. Tripathy, S.K., H.B. Black, E. Goldwasser, and J.M. Leiden. 1996. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat. Med.* 2: 545–550.

20. Yang, Y., G. Trinchieri, and J.M. Wilson. 1995. Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. *Nat. Med.* 1:890–893.

21. Zabner, J., B.W. Ramsey, D.P. Meeker, M.L. Aitken, R. Balfour, R.L. Gilson, J. Launspach, R.A. Moscicki, S.M. Richards, T.A. Standaert, et al. 1996. Repeat administration of an adenovirus vector encoding cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis. *J. Clin. Invest.* 97:1504–1511.

22. Tursz, T., A. Le Cesne, P. Baldeyrou, E. Gautier, P. Opolon, C. Schatz, A. Pavirani, M. Courtney, D. Lamy, T. Ragot, et al. 1997. Phase I study of a recombinant adenoviral-mediated gene transfer in lung cancer patients. *J. Natl. Cancer Inst.* 88:1857–1863.

23. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. *J. Immunol.* 126:1614–1619.

24. Venet, A., I. Bourgault, A.M. Aubertin, M.P. Kieny, and J.P. Levy. 1992. Cytotoxic T-lymphocyte response against multiple simian immunodeficiency virus (SIV) proteins in SIV-infected macaques. *J. Immunol.* 148:2899–2908.

25. Van Ginkel, F.W., C. Liu, J.W. Simecka, J.Y. Dong, T. Greenway, R.A. Frizzell, H. Kiyono, J.R. McGhee, and D.W. Pascual. 1995. Intratracheal gene delivery with adenoviral vector induces elevated systemic IgG and mucosal IgA antibodies to adenovirus and β-galactosidase. *Hum. Gene Ther.* 6:895–903.

26. Flomenberg, P., V. Piaskowski, R.L. Truitt, and J.T. Casper. 1996. Human adenovirus-specific CD8+ T-cell responses are not inhibited by E3-19K in the presence of gamma interferon. *J. Virol.* 70:6314–6322.

27. Mc Elvaney, N.G., and R.G. Crystal. 1995. IL-6 release and airway administration of human CFTR cDNA adenovirus vector. *Nat. Med.* 1:182–184.

28. Wesseling, J.G., G.J. Godeke, V.E. Schijns, L. Prevec, F.L. Graham, M.C. Horzinek, and P.J. Rottier. 1993. Mouse hepatitis virus spike and nucleocapsid proteins expressed by adenovirus vectors protect mice against a lethal infection. *J. Gen. Virol.* 74:2061–2069.

29. Gallichan, W.S., D.C. Johnson, F.L. Graham, and K.L. Rosenthal. 1993. Mucosal immunity and protection after intranasal immunization with recombinant adenovirus expressing herpes simplex virus glycoprotein B. *J. Infect. Dis.* 168:622–629.

30. Both, G.W., L.J. Lockett, V. Janardhana, S.J. Edwards, A.R. Bellamy, F.L. Graham, L. Prevec, and M.E. Andrew. 1993. Protective immunity to rotavirus-induced diarrhoea is passively transferred to newborn mice from naive dams vaccinated with a single dose of a recombinant adenovirus expressing rotavirus VP7sc. *Virology.* 193:940–950.

31. Xiang, Z.Q., Y. Yang, J.M. Wilson, and H.C. Ertl. 1996. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology.* 219:220–227.

32. Rodrigues, E.G., F. Zavala, D. Eichinger, J.M. Wilson, and M. Tsuji. 1997. Single immunizing dose of recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity against malaria. *J. Immunol.* 158: 1268–1274.