ABSTRACT

Previous studies with a mycobacterial heat shock protein (hsp-65) have demonstrated some efficacy using cationic liposome-mediated gene transfer in murine i.p. sarcoma models. To further analyze the efficacy of hsp-65 immunotherapy in clinically relevant models of localized cancer, immunocompetent mice bearing i.p. murine mesothelioma were treated with four i.p. doses of a cationic lipid complexed with plasmid DNA (pDNA) containing hsp65, LacZ, or a null plasmid. We observed >90% long-term survival (median survival, 150 days versus ~25 days, treated versus saline control, respectively) in a syngeneic, i.p. murine mesothelioma model (AC29). Long-term survivors were observed in all groups treated with lipid complexed with any pDNA. Lipid alone or DNA alone provided no demonstrable survival advantage. In a more aggressive i.p. model of mesothelioma (AB12), we observed >40% long-term survival in groups treated with lipid:pDNA complexes, again irrespective of the transgene. To ask whether these antitumor effects had led to an adaptive immune response against the tumor cell, we rechallenged long-term survivors in both murine models s.c. with the parental tumor cell line. Specific, long-lasting systemic immunity against the tumor was readily demonstrated in both models (AB12 and AC29). Consistent with these results, spleenocytes from long-term survivors specifically lysed the parental tumor cell lines. Depleting the CD8+ T-cells from the spleenocyte pool eliminated this lytic activity. Lipid:pDNA treatment of athymic, SCID, and SCID/Beige mice bearing a murine i.p. mesothelioma (AC29) resulted in only a slight survival advantage, but there were no long-term survivors. Treatment of immunocompetent mice depleted of specific immune effector cells demonstrated roles for CD8+ and natural killer cells. Although the exact mechanism(s) responsible for these antitumor effects is unclear, the results are consistent with roles for both innate and adaptive immune responses. An initial tumor cell killing stimulated by cationic lipid:pDNA complexes appears to be translated into long-term, systemic immunity against the tumor cell. These results are the first to demonstrate that adaptive immunity against a tumor cell can be induced by the administration of lipid:pDNA complexes. Multiple administrations of cationic lipid complexed with pDNA lacking an expressed transgene could provide a promising generalized immune-mediated modality for treating cancer.

INTRODUCTION

One of the major obstacles to successful cancer gene therapy has been the inability to deliver therapeutic transgenes effectively to the majority of tumor cells. For this reason, gene transfer approaches that generate antitumor immune responses are especially appealing, and many strategies have been developed using both viral and nonviral vectors (1) in which tumor cells have been transfected with various immunostimulatory transgenes, such as cytokines, MHC class I molecules, and co-stimulatory molecules. One principle that seems to be emerging from these studies is that repetitive immunization protocols will be needed to induce and maintain cytotoxic T-cell responses (2). Because the administration of viral vectors has been shown to induce powerful antiviral immune responses that impair repeat infection and expression (3–6), non-viral-based modalities for cancer gene therapy, such as cationic lipid-mediated gene transfer, have particular promise in the immunotherapy context. Potential advantages of nonviral vectors in immunotherapy applications include the simplicity of the approach, the inherent safety of delivering a nonreplicating plasmid vector, and most importantly, the feasibility of repeatedly delivering genes to tumors that might elicit the “danger signals” needed to activate the immune system and lead to the destruction of tumor cells (7).

Previous cancer gene therapy studies using cationic liposome-mediated gene transfer have examined the efficacy of specific transgenes, including herpes simplex virus thymidine kinase (HSVtk) in pancreatic cancer (8) and endothelial cells (9), tumor necrosis factor-α in a murine model of disseminated breast cancer (10), and allogeneic MHC class I molecules in melanoma (11, 12). Although several such studies have demonstrated limited in vivo efficacy, one particularly promising approach used cationic lipid-mediated gene transfer of a mycobacterium-derived heat shock protein (hsp-65) in a murine i.p. sarcoma model (13–15). In related experiments, Wells et al. (16) demonstrated that mice immunized i.p. with B16 cells expressing hsp-65 displayed significant resistance to a subsequent challenge with the parental B16 cells. The underlying immune mechanisms responsible for the antitumor effects observed with lipid:pDNA3 therapy, and more specifically with the use of the Mycobacterium leprae-derived hsp65 gene, remain unclear. Heat shock proteins can function as “molecular chaperones” and could potentially chaperone small antigenic peptides to MHC molecules for more efficient antigen presentation (17–20). In addition, bacterial and mycobacterial heat shock proteins are themselves extremely immunogenic molecules (21) and may provide an inflammatory milieu in the tumor cell environment.

To evaluate further the utility of hsp-65 immunotherapy, we studied the antitumor effects of cationic liposomes complexes with pDNA containing hsp65 delivered i.p. in a clinically relevant, localized model of cancer, namely, i.p. mesothelioma. We hypothesized that cationic lipid: pDNA complexes containing the hsp65 gene would elicit specific therapeutic efficacy compared with lipid:pDNA complexes containing the cDNA for either a nontherapeutic bacterial protein, e.g., βGal, or a plasmid carrying no transgene, i.e., a null plasmid. Accordingly, immunocompetent mice bearing macroscopic i.p. murine mesothelioma were treated with multiple i.p. doses of cationic lipid complexed with pDNA. Animals were examined for survival and long-term antitumor immunity. As postulated, we saw striking increases in survival as well as the induction of long-term adaptive immune responses in animals treated
with cationic lipid:phsp65 complexes. Surprisingly, however, we also found that equivalent responses could be obtained with our “control” lipid:pDNA complexes. These experiments demonstrated that repeated i.p. delivery of lipid:pDNA complexes, regardless of the transgene used, induced powerful antitumor responses that included total elimination of tumor burden for a significant proportion of animals and the development of long-term, antitumor immunity in these animals concomitant with the induction of tumor cell-specific cytotoxic CD8+ T-lymphocytes. Delivery of lipid:pDNA complexes thus could be a promising modality for treating localized malignancies such as malignant mesothelioma, ovarian cancer, brain tumors, or bladder cancer.

**MATERIALS AND METHODS**

**Cationic Lipids.** Genzyme cationic lipid no. 67 (GL-67; N4-spermine cholesterylcarbamate) was prepared by small molecule synthesis as described by Lee et al. (22). The neutral colipid DOPE and the stabilizing lipid DMPE-PEG3000 were obtained from Avanti Polar Lipids (Alabaster, AL). Solutions of GL-67, DOPE, and DMPE-PEG5000 were coformulated and lyophilized from r-butanol-water (90:10, v/v) at a molar ratio shown previously to be effective, namely (GL-67:DOPE:DMPE-PEG5000, 1:2:0.05, mol/mol/mol; Ref. 23). Prior to use, the lyophilized lipids were hydrated for 10 min with sterile water at 4°C and then vortexed for 2 min to generate liposomes. All pDNA was diluted in sterile water. After both lipid and pDNA were allowed to equilibrate under a stream of nitrogen as described previously (24). Residual CHCl3 was removed by lyophilization.

**Plasmids.** All plasmids were constructed using a backbone described previously (22, 25). The 5.6-kb pcMVhsp65 and pcMVgal plasmids contain the cDNA from the M. leprae-derived hsp65 and Escherichia coli-derived LacZ, respectively. The null plasmid (pNull) is identical to these cDNAs except that it has no transgene cDNA, i.e., it contains the cytomegalovirus promoter, hybrid intron, bovine growth hormone poly(A) sequences, and the kanamycin resistance gene (22, 25).

**Plasmid DNA.** Plasmid DNA was prepared by bacterial fermentation and purified by ultracentrifugation and sequential column chromatography. Purification was performed in a 15-liter Chemap fermenter at 37°C for 24 h using HCD media (Genzyme proprietary media) containing 100 μg/ml kanamycin. The purified preparations were predominantly supercoiled; exhibited spectrophotometric A260 nm/A280 nm ratios of 1.75–2.0; were free of detectable RNA; contained <10 μg of protein, 10 μg of chromosomal DNA, and 5 endotoxin units/mg of plasmid DNA (LAL assay; BioWhittaker), and were free of colony-forming units in a bioburden assay.

Expression of the hsp-65 gene product was confirmed in vitro by GL-67-mediated transfection of multiple cell lines (data not shown), followed by immunohistochemical detection using a monoclonal antibody specific for hsp-65 (4D6; StressGen Biotechnologies, Victoria, BC, Canada). Expression from the pcMVgal plasmid has been demonstrated previously (22, 25).

**Cell Lines.** AB12 and AC29 were murine mesothelioma cell lines (provided by Dr. Jay K. Kolls, LSU School of Medicine, New Orleans, LA). These cell lines were originally generated by Dr. Bruce Robinson (Queen Elizabeth II Medical Center, Perth, Australia) by i.p. implantation of asbestos fibers in BALB/c and CBA/J mice, respectively, and have been well characterized (26). These cells were cultured and maintained in high glucose DMEM (Mediatech, Washington, DC) supplemented with 10% FCS (Georgia Biotechnology, Atlanta, GA), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mm glutamine. The RENCA cells were murine (BALB/c) renal adenocarcinoma cells of spontaneous origin obtained as a gift from Dr. Kenneth Cowan (Medical Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, MD). Line 1 was a murine (BALB/c) bronchoalveolar carcinoma obtained as a gift from Dr. Steve Dubinett (UCLA, Los Angeles, CA). LLC cells originated as a carcinoma in the lung of a C57BL/6 mouse (27) and were purchased from the American Type Culture Collection. The murine lymphoma cell line YAC-1 (ATCC TIB-160), which has low levels of MHC class I and is constitutively sensitive to NK-cell-mediated lysis, has been described elsewhere (28, 29). RENCA, Line 1, LLC, and YAC-1 cells were cultured in RPMI 1640 with 10% FCS, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mm glutamine.

**Mice.** Female BALB/c, female CB17-SCID, and male CB17-SCID/Beige mice (6–8 weeks old; weight, ~25 g) were obtained from Taconic Laboratory (Germantown, NY). Male CBA/J mice (7–8 weeks old; weight, ~25 g) were obtained from the National Cancer Institute (Frederick Cancer Research & Development Center, Frederick, MD). Homozygous NCR nude mice (5–8 weeks old; weight, ~25 g) were also obtained from Taconic Laboratory. These athymic mice originally were derived from a BALB/c background. Animals were housed either in the animal facility at the Wistar Institute (Philadelphia, PA) or at Genzyme. The Animal Use Committees of the Wistar Institute and University of Pennsylvania or Genzyme approved all protocols in compliance with the Guide for the Care and Use of Laboratory Animals.

**i.p. Murine Mesothelioma Models.** Two independent intracavitary tumor models of murine mesothelioma (AC29 and AB12) were established to examine the treatment efficacy of locally administered liposome-pDNA complexes. Murine mesothelioma (AB12 or AC29) cells were grown to 80% confluence in culture flasks, the medium was aspirated, and cells were harvested using 0.05% trypsin-Versene (Life Technologies; Grand Island, NY). Cells were then pelleted (1000 rpm, 3 min) and resuspended at 1 × 106 cells/ml in serum-free DMEM for i.p. administration. Cell concentrations were determined by counting aliquots of the cell suspensions in a Coulter counter (Miami, FL). AB12 and AC29 cells (5 × 105/0.5 ml) were injected i.p. into BALB/c and CBA/J mice, respectively, using a 26-gauge needle. Approximately 5–8 days after tumor cell injection, macroscopic (0.5–1 mm) tumor nodules could be identified on the small bowel mesentery. Later, tumor could be observed on the diaphragm, peritoneal surface, porta hepatis, lesser sac, and retroperitoneum.

Survival studies were performed in lieu of tumor burden assessment because of the difficulty in harvesting tumor densely adherent to the abdominal viscera. The GL-67:pDNA complexes were combined in a 1:4 ratio using 50–100 μg pDNA, a dose previously found to be efficacious in i.p. murine sarcoma models (13). Treatments were initiated when ~1 mm tumor nodules were identified post tumor cell inoculation. Mice received four i.p. administrations of cationic lipid:pDNA complexes at 4 day intervals.

Following treatment, long-term survivors were anesthetized (xylazine/ketamine; A. J. Buck & Son, Inc, Baltimore, MD) and challenged s.c. in the flank with 5 × 105 cells of the parental tumor cell line (AB12 or AC29) in 150 μl of serum-free DMEM. An additional murine bronchoalveolar lung cancer cell line, Line 1 (syngeneic in BALB/c mice), was also used to challenge the long-term survivors in the AB12 model to evaluate the specificity of any antitumor response. Approximately 2 weeks after the s.c. challenge, flank tumors were excised and weighed; the animals were maintained to permit continuation of the survival study.

**Evaluation of T-Cell Responses to Lipid:pDNA Therapy.** Adaptive cellular immune responses to lipid:pDNA treatment were evaluated using a CTL assay. Pooled spleen cells (two to four mice per group) isolated from mice several months (see figure legends for specific time points) after i.p. treatment with lipid:pDNA complexes were cocultured in a volume of 2 ml in 24-well plates (5 × 105 cells/well in RPMI plus 10% FCS, 2-mercaptoethanol, and HEPES) with stimulator cells, i.e., 2 × 105 AB12 or AC29 cells treated with 100 μg/ml mitomycin C for 30 min at 37°C. Plates were incubated at 37°C in a 5% CO2 atmosphere for 6 days to expand tumor-specific CTLs. Effector cells were then harvested and pooled for further processing.

To deplete CD8+ T-cells from the expanded splenocyte population, aliquots of effector cells were incubated for 20 min at 4°C with a CD8+–specific murine antibody (LYT2; Dynal, Oslo, Norway) linked to magnetic beads. Beads and bound CD8+ T-cells were removed from the incubation medium with a magnet.

To measure cytotoxicity, untreated and CD8+–depleted effector cells were
incubated with $5 \times 10^5$ $^{51}$Cr-labeled target cells/well in triplicate for 5 h in a V-bottomed 96-well microtiter plate. Target cells were incubated with murine mAbs (clone R35–95; PharMingen) or an antibody to deplete the CD4$^+$ (clone GK1.5; PharMingen), CD8$^+$ (clone 53-6.7; PharMingen), or NK cell populations (antiasialo GM1; Wako Bioproducts, Richmond, VA). Dose levels were based on manufacturer’s recommendations for >90% depletion of the specific cell type. Depletion of each cell type to at least these levels was demonstrated at these doses (data not shown). Injections were administered for 3 consecutive days prior to inoculation with AB12 cells. Thereafter, a maintenance dose of antibody was injected i.v. every 3–4 days throughout the entire (16 day) treatment period to ensure depletion of the targeted cell type. Mice received four i.p. injections of lipid:pNull complex or saline as described above. At day 13 post cell (AB12) inoculation, one mouse from each group was sacrificed, and the splenocytes were harvested for fluorescence-activated cell-sorting analysis to quantify depletion of the specific immune effector cells; each targeted cell type was specifically and significantly depleted by this treatment (data not shown). The remaining mice in each group were followed for survival to evaluate the effects of depleting these immune cell types.

**Statistics.** Differences in tumor weights in the animal experiments were determined by one-way ANOVA. Post hoc comparisons of specific paired groups were performed using Fisher’s analysis. Statistical significance was set at $P < 0.05$. Kaplan-Meier survival curves were analyzed with the Mantel-Cox Log-rank test. Results are expressed as the mean ± SE.

**RESULTS**

The therapeutic effects of cationic lipid:pDNA treatment were evaluated in two i.p. mesothelioma models, AC29 and AB12. The mechanisms underlying these effects were probed as a function of time, using several methods. The functional involvement of different immune cells during treatment with complex was evaluated by using immunodeficient mouse strains and by depleting specific immune cell populations in normal animals prior to treatment. Treatment-generated long-term memory responses were characterized for the possible involvement of NK cells and cytotoxic lymphocytes by isolating splenocytes at times well after treatment and evaluating their ability to kill tumor cells in vitro.

**Treatment of Lp. Mesothelioma in the AC29 Tumor Model.** To assess the antitumor effects of cationic lipid:pDNA treatment, we first studied AC29 murine malignant mesothelioma cells growing within the peritoneal cavity of an immunocompetent host. Fig. IA depicts Kaplan-Meier cumulative survival in groups of CBA/J mice bearing syngeneic i.p. mesothelioma (AC29) that received four i.p. administrations of (a) saline, (b) cationic liposomes (GL-67$^+$) alone, (c) pHS65 alone, (d) GL-67 complexed with pHS65, or (e) GL-67 complexed with pGal. The pGal vector was used to delineate specificity of the hsp65 gene product and contained the same prokaryotic plasmid backbone as the pHS65 vector. Treatments were initiated on day 8 when macroscopic tumor nodules ~1 mm in size were identified; subsequent doses were delivered at 4-day intervals.

Animals treated with saline had a median survival of 28 days; all mice were dead by day 35. Administration of GL-67 alone or pHS65 alone had no effect on median survival and produced no long-term survivors. In contrast, administration of the combination of cationic lipid and pDNA (either pHS65 or pGal) resulted in a marked survival benefit, with all of the animals alive 84 days into the study ($P < 0.0001$). These treated animals also demonstrated no i.p. tumors when sacrificed on day 94. Of note was the observation that lipid complexed with pDNA containing either the bacterially derived LacZ transgene (pGal) or the mycobacterial hsp65 transgene were equally effective in producing long-term, tumor-free survivors, suggesting the lack of any transgene-specific effect.

A second survival experiment in the AC29 model was designed to determine (a) whether the observed antitumor effects were specific to the GL-67 liposome, and (b) whether a similar pDNA containing no transgene at all (pNull) would have the same effect. Tumor-bearing animals were treated with (a) saline, (b) GL-67 complexed with pNull, (c) GL-67 complexed with pHS65, and (d) DC-Chol:pHS65 complexes. Long-term survivors in B were challenged with AC29 cells s.c. as described in “Material and Methods” (arrowhead). Control mice received 0.9% NaCl (♦) at each dose interval. Significant long-term survival (>90%) was observed only in treated mice receiving lipid:pDNA complexes, namely, GL-67:pHS65 (A and B), GL-67:pGal (A), and DC-Chol:pHS65 (B).
were no long-term survivors in the control, saline-treated group. There was no statistically significant difference ($P = 0.3776$) between the survival curves using phs65 or pNull complexed with lipid. Untreated animals reproducibly died by 25–30 days (not shown) as did the saline control group. Plasmid DNA alone (phs65) offered a small survival benefit compared with the saline control, but this difference was not statistically significant.

To determine whether the long-term survivors (see Fig. 2A) had developed specific antitumor immunity, they were challenged s.c. in the flank on day 90 with the parental tumor AB12. On day 113, these tumors were excised and weighed. On day 119, these same animals were challenged s.c. with a control, syngeneic tumor cell line, Line 1 (a murine bronchoalveolar lung cancer). For each tumor cell challenge, naive mice received injections of with these same tumor cell lines as controls. Fig. 2B shows that compared with the mass of AB12 tumor harvested from the flanks of control mice (0.27 ± 0.08 g), there was essentially no tumor growth ($P < 0.01$) in those mice that had been treated previously with GL-67:phs65 (0.02 ± 0.01 g) or GL-67:pNull (0.02 ± 0.01 g). In contrast, Fig. 2B also shows that the flank challenge with Line 1 cells yielded equivalent-sized tumor nodules on the flanks of all mice tested, implying that specific and systemic antitumor immunity to AB12 had developed as a result of the lipid: pDNA treatment.

**Lipid:pDNA Dose Response in the AB12 Tumor Model.** We examined the dose response to lipid:pDNA treatment in the AB12 model. In a treatment scheme identical to the previous AB12 experiments (see above), BALB/c mice bearing i.p. AB12 tumors were treated with four doses of lipid complexed with pβGal at a GL-67: pβGal ratio of 1:4 (mol/mol) and at increasing pDNA dose, i.e., 0, 10, 25, and 100 μg. Fig. 3 demonstrates that no therapeutic effect was demonstrable at a dose of 10 μg pDNA, but that a 40% long-term survival rate could be achieved at a dose of 100 μg pDNA ($P = 0.0002$). Lipid complexed with 25 μg of pβGal showed an intermediate survival advantage (20% at 70 days) that was significant compared with saline controls ($P = 0.0077$) but was not significant when compared with the 100-μg group ($P = 0.2928$). A similar dose response to lipid:pDNA complexes was observed in this model when the phs65 vector was used (data not shown). Thus, these data demonstrate that i.p. AB12 tumor cells could be eliminated with lipid:pDNA complex treatment in a dose-dependent fashion, and that this elimination was independent of the transgene.

![Fig. 3. Dose response to lipid:pDNA complexes administered i.p. in BALB/c mice (n = 10/group) bearing i.p. murine mesothelioma (AB12). Mice received i.p. administrations of GL-67 complexed with pβGal (1:4 ratio) on four separate occasions (arrows). Plasmid DNA doses were 0, 10, 25, or 100 μg and were complexed with appropriately increasing amounts of lipid to maintain a 1:4 ratio. No significant therapeutic effect was observed with 10 μg pβGal (C) compared with a saline (B) control. Improved survival was best demonstrated in mice that received lipid complexes containing 100 μg pβGal (D; $P = 0.0002$), with 40% survival at 70 days.](image)

To determine whether a protective antitumor immune response had been generated as a result of treatment, the long-term surviving animals were rechallenged on day 115 (Fig. 1B, arrowhead) with a s.c. injection of $5 \times 10^6$ AC29 cells into each flank. Tumor growth in these long-term survivors was compared with that in naive (untreated) CBA/J mice injected with the same number of tumor cells. After 18 days, all untreated mice had large flank tumors, whereas none of the previously treated mice had detectable flank tumors. This result was independent of the cationic lipid or pDNA used for treatment. Thus, in the AC29 model, animals treated with lipid:pDNA complexes were both “cured” of their disease and exhibited long-term protection against a distal challenge by these same tumor cells.

**Treatment of i.p. Mesothelioma in the AB12 Tumor Model.** To determine whether lipid:pDNA complexes could provide therapeutic effects in another intracavitary tumor model in a different genetic background, we treated immunocompetent BALB/c mice bearing a second syngeneic i.p. murine mesothelioma, namely AB12. Animals received four i.p. administrations of cationic liposome (GL-67) complexed with 100 μg of pDNA (either phs65 or pNull) every 3 days starting at day 5. Although the long-term survival of treated animals in this model (Fig. 2A) was somewhat less impressive than that seen in the AC29 model, it should be noted that AB12 tumors are more aggressive and resistant to most chemotherapeutic and gene therapy treatment modalities. Nonetheless, a significant percentage (30–50%) of animals survived long term (150 days) when mice were treated with GL-67 complexed with either phs65 or pNull; there

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5 Unpublished results.
Lipid:pDNA Therapy in Immunodeficient Murine Models bearing AC29 Tumors. To further investigate the immune mechanisms responsible for the improved survival observed in animals treated with lipid:pDNA, survival studies were performed in immunodeficient murine models. Four i.p. administrations of GL-67:hsp65 complexes or saline were evaluated in three different immunodeficient mouse strains bearing i.p. murine mesothelioma (AC29) as described in “Materials and Methods.” Kaplan-Meier survival analysis demonstrated incremental improvements in median survival in athymic (~10 days; A), SCID (~10 days; B), and SCID/Beige (~5 days; C). Therapeutic effects were similar in the SCID model (B) when lipid was complexed with pDNA containing no transgene (pNull, ◦), hsp65 (○), or βGal (□) transgenes.

Fig. 4. Effects of lipid:pDNA treatment in immunodeficient mouse strains. GL-67: hsp65 complexes (50 μg of pDNA) were administered i.p. in four doses (arrows) to athymic (A), SCID (B), and SCID/Beige (C) mice (n = 12/group) bearing murine mesothelioma (AC29) as described in “Materials and Methods.” Kaplan-Meier survival analysis demonstrated incremental improvements in median survival in athymic (~10 days; A), SCID (~10 days; B), and SCID/Beige (~5 days; C). Therapeutic effects were similar in the SCID model (B) when lipid was complexed with pDNA containing no transgene (pNull, ◦), hsp65 (○), or βGal (□) transgenes.

Effects of Immune Cell Depletion on Survival. To gain additional insight into the roles played by CD4+ and CD8+ T cells and NK cells as a result of lipid:pDNA treatment, the immunocompetent mice were depleted of these specific immune effector cells before treatment. Fig. 5 shows that in the AB12 model, lipid:pDNA complex treatment of CD8− or NK-cell-depleted animals was significantly less effective (all animals had died by day 13) than the same treatment in fully immune competent BALB/c mice. In fact, these CD8− or NK-cell-depleted animals died before untreated tumor-bearing animals. These results argue that both CD8+ and NK cells play important roles during the induction phase of treatment, leading to a slowing of tumor growth, the elimination of tumor cells, or both. The potential role played by CD4+ cells is somewhat less clear. Fig. 5 shows that ~50% of the CD4+−depleted animals survived long term. Although there is no statistical difference between the CD4−−depleted group and the treated group, the CD4−−depleted group may have survived marginally better (P = 0.16, Log-rank) than the untreated animals. Although the quantitative results of these immune cell depletion experiments may not be directly comparable to the results obtained in the immunodeficient mouse strains because they used different models, i.e., AB12 and AC29, respectively, the data are consistent with CD8+ and NK cells being critical to the long-term survival of the animals.

Evaluation of Cytotoxic Antitumor Cell Responses to Lipid: pDNA Therapy in the AC29 Tumor Model. In the AC29 model, challenging long-term survivors with a s.c. inoculation of AC29 cells led to the elimination of those cells (see above). We used in vitro cytotoxicity assays to ask whether CTLs or NK cells could have played a role in tumor cell rejection. To determine whether tumorspecific CTLs had been generated as a consequence of treatment, spleen cells were isolated from survivors in the experiment depicted in

Fig. 5. Effects of treatment in the AB12 model in BALB/c mice depleted of specific immune cell types. Antibodies specific for CD8+, CD4+, and NK cells were used to deplete these specific cell types before treatment with GL-67:pNull (arrows). In fully immunocompetent animals, the untreated group (□) had a median survival of ~20 days, whereas the treated group (●) had ~65% survivors at day 57. Depletion of NK (△) or CD8+ (○) cells led to decreases in median survival (median, 9–14 days), whereas CD4+ (○) depletion resulted in a median survival similar to that of untreated animals.
Fig. 1B at day 158 and cultured in vitro for 6 days with mitomycin-treated stimulator cells (AC29 cells). Spleen cells from naive and untreated (but tumor-bearing) CBA/J mice were processed similarly. Splenocytes were then tested for cytotoxicity against the parental tumor (AC29), a control murine cell line, LLC, and YAC-1 cells as targets. The murine lymphoma cell line, YAC-1, expresses virtually no MHC class I and is sensitive to NK-cell-mediated lysis (30). Allogeneic LLC cells were used because no other syngeneic murine tumor cell line was available for CBA/J mice. Fig. 6 shows that splenocytes isolated from long-term surviving mice treated with lipid: pDNA complexes demonstrated significant cytotoxicity for AC29 cells, whereas splenocytes from untreated mice bearing i.p. AC29 for at least 2 weeks did not. Fig. 6B shows that, as expected, no cytotoxicity could be demonstrated against the control LLC target cells using splenocytes from treated or untreated mice. Stimulated spleen cells from naive CBA/J mice, i.e., bearing no tumor, also showed no target cell lysis (AC29 or LLC; data not shown). Splenocytes from treated animals also exhibited significant lysis of YAC-1 cells (40% at an E:T ratio of 60:1). Together, these data indicate that cationic lipid:pDNA therapy produced long-term surviving animals that had generated cytotoxic lymphocytes specific for the AC29 tumor cells, that this induction was independent of the cationic lipid and plasmid used, and that there were significant numbers of activated NK cells present at this time point.

Evaluation of Cytotoxic Antitumor Cell Responses to Lipid:pDNA Therapy in the AB12 Tumor Model. Because treatment with lipid:pDNA complexes appeared to induce specific T-cell-mediated antitumor immunity in the AC29 model, we also investigated the relationship between long-term protection and T-cell-mediated cytotoxicity in the AB12 model. Spleen cells from long-term survivors that had been treated with GL-67:p hsp65 complexes [GL-67:pNull (○), GL-67:p hsp65 (△), or DC-Chol:p hsp65 (‰)] demonstrated activity against AC29 cells (△) but not LLC (○) cells. Stimulated splenocytes from naive CBA/J mice elicited no cytotoxicity for either the AC29 or LLC targets (data not shown). Data shown are representative of two studies.

Fig. 7A, spleen cells (three spleens/group) were isolated at day 91 and stimulated for 6 days in culture with mitomycin-treated AB12 cells and then tested for their ability to lyse the parental AB12 cell line (□) and two additional syngeneic targets, RENCA (○) and Line 1 (△). Only AB12 cells were lysed. Data shown are representative of four similar experiments. B, depletion of CD8+ T-cells from the stimulated splenocytes resulted in the complete elimination of cytotoxic activity against the parental AB12 cells (□). Stimulated splenocytes from naive BALB/c mice and from mice bearing i.p. AB12 without treatment showed no cytotoxicity for any of the targets AB12, RENCA, or Line 1 (data not shown).
exhibited a significant ability to lyse AB12 cells but showed virtually no cytotoxicity against the syngeneic control target cells, RENCA and Line 1. Specifically depleting CD8+ T-cells from the splenocyte pool (see “Materials and Methods”) completely eliminated this cytotoxic activity, as shown in Fig. 7B. Splenocytes from saline-treated mice bearing i.p. AB12 demonstrated minimal cytotoxicity (<10%) toward the parental cell line AB12 and no lysis of RENCA or Line 1 (data not shown). As expected, splenocytes isolated from naive BALB/c mice and stimulated with AB12 cells failed to lyse any of the targets tested (data not shown). Taken together, these data demonstrate that treating tumor-bearing animals with lipid:pDNA complexes resulted in the generation of CD8+ T cells specific for the tumor cell present in the peritoneal cavity during treatment. These data also suggest that CD8+ T cells were largely responsible for the rejection of the s.c. challenge because their elimination resulted in essentially a complete loss of cytotoxicity against AB12 cells (Fig. 7B).

We also evaluated the possible involvement of NK cells in animals that survived long term as a result of treatment with lipid:pDNA. Spleen cells were isolated from long-term surviving mice in an AB12 experiment (data not shown) at day 91 post tumor cell inoculation. In this experiment, groups of mice had been either untreated or treated with complexes of GL-67:pHsp65 or GL-67:pβgal. Untreated mice were all dead by day 21, whereas the latter two groups had 50 and 20% long-term survivors, respectively. Specific NK-cell-mediated target cell lysis was measured indirectly by incubating the cultured splenocytes (effectors) for 45 min with an excess of unlabeled YAC-1 cells prior to incubating them with 51Cr-labeled AB12 target cells (30). A comparison of Fig. 8A and Fig. 8B shows that in the presence of YAC-1 cell inhibition, there was at most a marginal (~10%) decrease in target cell lysis (60:1 E:T). As a positive control for the effectiveness of YAC-1 inhibition of NK-cell-mediated lysis, the lysis of 51Cr-labeled YAC-1 cells by these stimulated splenocytes could be inhibited completely by first incubating the splenocytes with unlabeled YAC-1 cells (data not shown). Equivalent levels of target cell (AB12) lysis were observed in animals receiving multiple treatments of either GL-67:pHsp65 or GL-67:pβGal complexes. In agreement with the data obtained using CD8+ cell depletion (Fig. 7), these results suggest that although they cannot be totally excluded, NK cells are not likely to play a major role in the direct, cell-mediated killing of the tumor cells in a challenge in long-term, immunized animals.

**DISCUSSION**

In this study, we have demonstrated that repeated i.p. administrations of a complex consisting of cationic lipid and bacterial plasmid DNA results in powerful effects against mesothelial tumors actively growing in the peritoneal cavity. Indeed, i.p. treatment with lipid:pDNA complexes resulted in long-term surviving animals in both the AC29 (Fig. 1) and AB12 (Figs. 2 and 3) models. Treatment was seen to produce long-term survivors that were not only free of tumor, but more importantly, were also specifically protected against a later rechallenge by the tumor cell present during treatment, i.e., a “memory” response had been generated as a result of the treatment. Induction of this specific, long-term protective immunity was shown to be paralleled by the generation of tumor-specific cytolytic CD8+ T cells. For example, splenocytes from long-term survivors in the AB12 model could kill AB12 cells but not syngeneic RENCA or Line 1 cells (Fig. 7). The splenocytes responsible for this killing appeared to be CD8+ because their elimination abolished this cytolytic activity (Fig. 7).

**Optimal Efficacy Is Obtained with DNA in a Complex.** An important observation from this study was that no significant therapeutic benefit was observed when DNA alone or lipid alone was administered to the tumor-bearing animals (Figs. 1 and 2). Antitumor activity was seen only when the DNA was first complexed with cationic liposomes. Similar findings have been seen in a study of i.v.-administered lipid:pDNA complexes using models of metastatic disease (31). Thus, our observed effects, and those of Dow et al. (31), are not simply due to the presence of bacterial DNA but seem to require a lipid:pDNA complex. Among the potentially unique functions of such a complex (over free pDNA) are that it may (a) serve to protect the DNA from degradation, (b) enable more effective uptake of the DNA by the relevant cells, or (c) enhance the delivery of the DNA to the cytoplasm or nucleus.

**Treatment Activates Both Innate and Adaptive Arms of the Immune System.** These results are consistent with a biphasic immune response to cationic lipid:pDNA complexes that consists of an immediate, innate response that then matures into an adaptive, memory-based response featuring cytotoxic lymphocytes specific for the tumor cell present during treatment. In this view, the initial, innate phase of the immune response is a consequence of the multiple
treatments with complex, and results in the elimination of a large proportion of tumor cells already established in the peritoneal cavity. This cytolytic phase of the response may serve to decrease the i.p. tumor cell load to the point where a later adaptive response can effectively eliminate any remaining tumor cells. We would thus interpret the results in the AC29 model as indicating that multiple administrations of complex was a very effective regime for generating these overlapping innate and adaptive cytolytic responses, and that these responses were somewhat less effective in the AB12 model, where not all animals survived long term. The immunodeficient animal studies (Fig. 4) and the antibody depletion studies (Fig. 5) suggest that the early, innate phase of the response is characterized by both CD8+ and NK cell involvement. It is likely that neutrophils, macrophages, and eosinophils also play a role in this early response.

This initial, cytotoxic response to treatment leads to an adaptive immune response characterized by tumor-specific CTLs. These CTLs are entirely capable of eliminating a s.c. challenge by tumor cells at a time point that is well removed from treatment. For example, the challenge time points shown in the AB12 model in Fig. 2 were at least 3 months after the final treatment. This long-lasting memory response is systemic in nature, as demonstrated by its ability to eliminate tumor cells implanted at a site distal to the original i.p. treatment. NK cells did not appear to play a major direct role in this systemic immunity because inhibiting NK-mediated killing had at most a minimal effect on tumor cell killing by splenocytes from immune animals (Fig. 8). This result was not unexpected because these mesothelioma models are reported to express high levels of MHC class I molecules (32), which would make them inappropriate targets for NK cells. However, these results do not exclude an important role for NK cells in this memory phase of the response (i.e., by the secretion of cytokines), they simply point to a minimal role for NK cells in the direct killing of the tumor cells.

Our observations that lipid:pDNA complexes can inhibit tumor growth, i.e., the early innate, cytolytic phase of the response, are consistent with several recent reports. For example, systemic administration of lipid:pDNA complexes lacking a transgene has been shown to reduce the tumor burden in several models of lung metastases (31). This inhibition of tumor growth was found to be dependent on NK cell activity and production of IFN-γ, and is entirely consistent with the role found for NK cells in the early, cytotoxic phase of the immune response in the present study (see Fig. 5). Similarly, complexes of cationic lipid, protamine, and pDNA have been seen to inhibit tumor growth in both lung and s.c. tumor models (33, 34). In this case, inhibition was correlated with the generation of the proinflammatory cytokines tumor necrosis factor-α, IFN-γ, and IL-12, which in turn were a consequence of the bacterial source of pDNA and its immunostimulatory CpG sequences (35–39). Finally, multiple peritumoral injections of CpG-containing oligodeoxynucleotides themselves, i.e., in the absence of a cationic DNA-condensing agent, have been shown to provide antitumor effects in an established s.c. neuroblastoma model (40). These effects were also found to be dependent on NK cells but did not appear to recruit CD8+ T cells into the tumor. By contrast, in the present study, immune cell infiltration into tumor was observed at early time points, using immunohistochemistry, in response to each treatment and consisted of macrophages, neutrophils, and T lymphocytes (data not shown). Thus, this early phase of the response to bacterial CpG sequences, delivered with or without a cationic, DNA-condensing agent, appears to involve multiple components of the innate immune system and can significantly inhibit tumor growth.

It is important to note, however, that the present results extend significantly the scope of these previous findings by demonstrating that this innate, cytolytic phase of the response can translate into the induction of a memory-based T-cell response that is systemic in nature and capable of eliminating a tumor cell challenge months after the initial treatment, i.e., a state of antitumor immunity is present. We are aware of no other such demonstration of the generation of systemic antitumor immunity by the administration of bacteria-derived DNA, either by itself, or as a complex with a cationic condensing agent.

A Possible Mechanism Based on Danger Theory. The exact mechanisms by which the initial treatment-induced inflammatory reaction and innate immune system involvement are converted into long-term adaptive immunity are not totally clear (41–43). One potential scenario is that these complexes lead to the lysis of some tumor cells by components of the innate immune system, resulting in the release of tumor antigens, perhaps bound to heat shock proteins (44, 45). Tumor antigen release at the site of immune stimulation could allow cross-priming of antigen-presenting cells such as dendritic cells or macrophages, from which the long-term, tumor-specific adaptive immune response described here could be generated (46, 47). Indeed, a recent study noted the existence of receptors on antigen-presenting cells for heat shock protein:peptide complexes (48) that may provide a path for antigens into the class I presentation pathway. According to the “danger theory” (7), tumor regression requires both T-cell activation and the presence of a second stimulus to perpetuate the T-cell response against tumor antigen. In this danger model, the response would proceed only as long as danger signals are present (7). We postulate that the repeated local administration of the lipid:pDNA complexes provides the danger signals necessary to enhance tumor cell lysis and induce CTL-mediated antitumor immunity.

Bacterial CpG Sequences Are Likely to Play a Role in Efficacy. It is likely that recognition of the bacterial CpG motifs in the plasmid DNA used in this study by components of the innate immune system are important for the generation of the subsequent immune responses. The immunogenicity of prokaryotic, i.e., plasmid, DNA in mammals is now well established and appears to be due in large part to the presence of immunostimulatory motifs consisting of unmethylated CpG dinucleotides. These CpG motifs have been found to trigger innate immune responses in mammalian hosts, characterized by the production of a number of cytokines, including IL-6, IL-12, and IFN-γ (35–39). Conversely, a significant reduction in the immunogenicity of bacterial DNA has been observed by methylating these CpG motifs (49). Indeed, in the mesothelioma models described here, mammalian DNA did not have the efficacy of bacterial pDNA (data not shown).

Other, nonexclusive mechanisms for the effectiveness of this therapy are also possible. Cationic lipid:pDNA complexes have been shown to up-regulate the expression of MHC class I molecules on tumor cells (11), which could enhance their subsequent recognition by CTLs. In addition, the introduction of double-stranded DNA (independent of the presence of CpG motifs) into non-immune cells has also been shown to increase the expression of genes necessary for antigen processing, such as proteasome proteins, transporters of antigenic peptides, and the co-stimulatory molecule B7.1 (50).

In conclusion, repeated i.p. administrations of lipid:pDNA complexes appear to generate powerful and specific antitumor immune responses that lead to a significant cure rate. Immunologically, the state of antitumor immunity that develops appears to be the overall product of an initial antitumor response of the innate immune system in response to plasmid DNA presented in the context of a cationic liposome that in turn stimulates the generation of a specific adaptive immune response against the tumor cell. These data are the first to support the idea that treatment with lipid:pDNA complexes can induce a state of antitumor immunity in the treated animal, and as such...
have significant implications for the treatment of cancer and its metastases.

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Cationic Lipid:Bacterial DNA Complexes Elicit Adaptive Cellular Immunity in Murine Intraperitoneal Tumor Models

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