Antibody Targeting of Doxorubicin-loaded Liposomes Suppresses the Growth and Metastatic Spread of Established Human Lung Tumor Xenografts in Severe Combined Immunodeficient Mice

Masahiko Sugano, Nejat K. Egilmez, Sandra J. Yokota, Fang-An Chen, Jennifer Harding, Shi Kun Huang, and Richard B. Bankert

Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York 14263 [M. S., N. K. E., J. J. Y., F.-A. C., R. B. B.]; and Alza Corporation, Mountain View, California 94025 [J. H., S. K. H.]

ABSTRACT

β1 integrins, expressed on the cell surface of human non-small cell lung carcinomas, are used here as a target for the selective delivery of anticancer drug-loaded liposomes. Fab’ fragments of a monoclonal antibody specific for human β1 integrins were conjugated to sterically stabilized liposomes. Confocal microscopy of β1 integrin-positive lung tumor cells incubated with fluorescently labeled anti-β1 Fab immunoliposomes revealed a tumor-specific binding and efficient internalization of the liposomes into the tumor cells. The ability of these liposomes to deliver cytotoxic drugs to the tumor and kill these cells was demonstrated in vitro by incubating tumor cells with doxorubicin-loaded anti-β1 Fab’ immunoliposomes. The drug-loaded immunoliposomes were >30-fold more cytotoxic to the tumor cells than drug-loaded liposomes without antibody, nonspecific Fab’ control immunoliposomes with drug or immunoliposomes without drug. The therapeutic efficacy of doxorubicin-loaded immunoliposomes was also evaluated in a metastatic human lung tumor xenograft/severe combined immunodeficient (SCID) mouse model. SCID mice that received i.v. injections of human lung tumor cells developed primary tumor nodules in the lung that subsequently metastasized to the liver and adrenal gland. Treatment of SCID mice bearing established lung tumor xenografts with doxorubicin-loaded anti-β1 Fab’ immunoliposomes resulted in a significant suppression of tumor growth (monitored periodically by quantifying serum levels of a tumor marker), whereas tumors grew progressively in mice treated with control formulations. In addition to suppressing the growth of the primary lung tumor nodules, the immunoliposomes prevented the metastatic spread of the tumor to the liver and adrenal glands and increased the median survival time of the tumor-bearing mice. We conclude that Fab’ immunoliposomes directed to tumor-associated integrins represent a potentially viable approach clinically for the selective delivery of drugs to solid tumors and may be useful in preventing the metastatic spread of lung cancer.

INTRODUCTION

Lung cancer carries the highest mortality rate among cancer patients in both men and women. In disseminated stage IV disease, there is no effective curative therapy (1). Curative surgery is the preferred therapy in localized non-small cell lung cancer cases. However, postoperative failure rates are 30–40% for stage I disease and 70–90% for stage II disease. Median survival for stages III and IV range from less than 12–18 months. Clearly, new and more effective adjuvant therapies are needed to prevent the reoccurrence and spreading of this disease. One approach to achieving increased therapeutic effective-

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2 Present address: Juntendo University School of Medicine, First Department of Surgery, Tokyo, 113-8421 Japan.

3 Present address: Department of Otolarngology, Division of Head and Neck Surgery, New York University Medical Center, New York, NY 10016.

4 To whom requests for reprints should be addressed, at Department of Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-8668; Fax: (716) 845-8906; E-mail: bankert@sc3101.med.buffalo.edu.

5 The abbreviations used are: PEG-DSPE, polyethylene glycol-derivatized distearoyl phosphatidylethanolamine; SCID, severe combined immunodeficient; PSA, prostate-specific antigen.

ness of adjuvant chemotherapy while at the same time reducing the side effects associated with cytotoxic drugs is to use liposomes as drug carriers. Improved liposome formulations, particularly liposomes containing components such as phosphatidylinositol, monosialoganglioside, or PEG-DSPE5 have significantly improved their efficacy. These sterically stabilized liposomes (Stealth liposomes) avoid rapid clearance by the reticuloendothelial system, thereby increasing their time in circulation (2, 3). They have been used successfully to deliver chemotherapeutic agents such as doxorubicin, epirubicin, and vincristine to mouse colon or mammary tumors and have been shown to enhance therapeutic activity compared with the same drugs administered in the free form or entrapped in conventional (rapidly cleared from circulation) liposomes (4–7). Other studies have shown enhanced therapeutic effects of sterically stabilized liposome drug delivery to human lung tumor xenografts in SCID mice (8) and human prostate (9) and ovarian (10) tumor xenografts in nude mice.

The use of antibodies to direct the delivery of drugs carried inside of liposomes to tumors represents a potentially viable method for further increasing the specificity and enhancing the therapeutic effect of liposome drug delivery (11, 12). Although many laboratories have demonstrated that antibodies can be used to enhance the selective liposome drug delivery to tumors in vitro (13–17), demonstrating this in vivo has proven to be a far more daunting task (18, 19). The problem in vivo is likely attributable to many factors, but three obvious pitfalls stand out, i.e., the inability of the targeted liposomes to gain access to the tumor, the aggregation and subsequent premature clearance of immunoliposomes by the reticuloendothelial system, and the failure of the immunoliposomes that bind to the tumor target to enter the cell and/or release their drug. The development of methods for coupling specific ligands to the PEG terminus of Stealth liposomes (20–25) and the use of Fab’ fragments of antibodies instead of complete immunoglobulin molecules to conjugate to the liposomes (25) have increased the time in circulation and improved the extravasation of the immunospecific Stealth liposomes into solid tumors in vivo. This new generation of immunospecific Stealth liposomes targeted to a cell surface integrin has been tested here and shown to be effective in suppressing the growth and metastasis of a human lung tumor in SCID mice.

MATERIALS AND METHODS

Mice. CB-17 scid/scid mice were obtained from our breeding colony. All mice were maintained in microisolation cages (Lab Products, Femersburg, MA) under pathogen-free conditions. Animals of both sexes were used in the studies at 8–12 weeks of age.

Antibodies. Monoclonal antibodies 1F11 and 2C3, both mouse IgG1, were developed in our laboratory using technology described previously (26, 27). 1F11 is specific for the human integrin β1 subunit, and 2C3 is specific for the hapten phthalate. For preparation of the (Fab’)2 fragments, purified antibodies...
were diazoyed against 0.2 M acetic buffer (pH 4.0), concentration was adjusted to 2 mg/ml, and pepsin was added to a final concentration of 0.2 mg/ml. Digestion was performed at 37°C for 7 h, and the reaction was terminated by the addition of 0.1 volume 2 M Tris-base (pH 8). The reaction mix was diazoyed against PBS (pH 8) and was passed through a protein-G column equilibrated with the same buffer. Unbound fractions were collected, sterilized by filtration through a 0.22 μm filter, and the protein was collected by filtration.

**Liposomes.** A long-circulating liposome formulation of doxorubicin was prepared and characterized at ALZA Corp. (Mountain View, CA; formerly Sequus Pharmaceuticals, Inc.) using hydrogenated soy phosphatidylcholine (Natherman Phospholipids, Cologne, Germany), cholesterol (Credo, Fullerton, CA), PEG-DSPE, and N-(3-trifluoroacetyl) liposomes (Shearwater Polymers, Huntsville, AL) in HEPES buffer (pH 7.2), after which the reducing agent was removed by passing the sample over a PD-10 desalting column (Pharmacia, Piscataway, NJ) pre-equilibrated with the same buffer without β-mercaptoethanol. The freshly reduced Fab' fragment was reacted with 5 molar excess of maleimide-PEG-DSPE (Shearwater Polymers, Huntsville, AL) in HEPES buffer (pH 7.2) at 37°C for 5 h. The conjugate was mixed with liposomes at a ratio that should yield ~10 Fab' molecules/liposome assuming that 25% of the Fab' conjugated to Mal-PEG-DSPE and 100% incorporated into the liposomes. The appropriate volume of liposomes was added to the Fab'-PEG-DSPE conjugate and was incubated overnight at 37°C. The next day, the unreacted maleimide was reduced by incubation with 2 molar β-mercaptoethanol for 30 min at room temperature. The Fab'-conjugated liposomes were purified by size exclusion chromatography on a 1.5 × 30-cm, A50m column (Bio-Rad, Hercules, CA) equilibrated with 25 mM HEPES/0.9% saline (pH 7.2). The amount of protein conjugated to the liposomes was quantitated by SDS-PAGE on a 4–20% gradient gel (Bio-Rad Laboratory, Hercules, CA). The protein in the gel was visualized with Sypro Orange (Bio-Rad, Hercules, CA), and the intensities of the samples were quantitated using an Alpha Innotech Corporation (San Leandro, CA).

**Tumor Cells.** The human lung squamous carcinoma cell line RPCI-2E9/IV4/PSA, which is adapted for rapid orthotopic growth in the SCID mouse lung and is genetically modified to secrete human PSA, was described by us previously (30).

**In Vitro Binding and Internalization Studies.** RPCI-2E9/IV4/PSA cells were fixed with 2% paraformaldehyde for 10 min in a humid chamber. Cells were washed with PBS, fixed with 2% paraformaldehyde–acetone for 7–10 min at room temperature, rinsed with water and were mounted with Polymount. Samples for confocal microscopy were prepared either prior to transfer to 37°C (time 0) or after incubation at 37°C (time 60 min). Images were obtained using a Bio-Rad MRC-600 laser scanning confocal microscope with an argon laser. The GHS filter set, with an excitation filter at 512 nm and an emission filter at 540 nm, was used to collect 1 μm optical sections. Projections of cells were displayed using Bio-Rad software.

**In Vitro Toxicity Studies.** RPCI-2E9/IV4/PSA cells were plated in 96-well culture plates (1 × 10^4 cells in 200 μl of culture medium/well) with the indicated quantity of liposome formulations. Cells were incubated at 37°C, 5% CO₂ for 5 days, and inhibition of cell growth was determined by the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (31).

**In Vivo Therapy Studies.** All mice received i.p. injections of the monoclonal antibody TM21 (kindly supplied by Dr. T. Tanaka of Tokyo Metropolitan Institute for Medical Science, Tokyo, Japan) to deplete the murine natural killer cells 1 day prior to tumor inoculations (day −1) as described (32). Mice were bled through the tail vein weekly starting at week 4 and ending at week 7. With the exception of the survival experiment, all mice were sacrificed at week 8 and were analyzed for tumor burden. Lung tumor burden was determined by counting the number of visible tumor nodules under a dissecting microscope. For the determination of liver and adrenal gland tumor weights, livers and adrenal glands of mice in the experimental groups were removed, weighed, and averaged for each group. The average liver and adrenal gland weights of age and sex-matched healthy controls (five mice) were then subtracted from the experimental groups, and the difference was plotted as tumor weight. PSA ELISA. The ELISA for the quantification of serum PSA levels was described by us previously (30). Briefly, 96-well plates were coated with a rabbit anti-HuPSA antibody (1 μg/ml in PBS) at 4°C overnight and washed with PBS containing 0.1% Tween 20. Plates were blocked with 1% BSA in borate-buffered saline at room temperature for 1 h and then washed with PBS containing 0.1% Tween 20. Mouse sera and PSA standards (0–4000 pg/ml) were added to the plates, followed immediately by the addition of 50 μl of a biotinylated mouse anti-HuPSA (100 ng/ml), and the plates were incubated for 2 h at room temperature. Plates were washed four times, 50 μl of streptavidin-conjugated horseradish peroxidase (1 μg/ml Sigma A3151) were added, and the samples were incubated at room temperature for 20 min. Plates were washed, and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma T8540) was added. Reaction was stopped by the addition of 100 μl of 0.1 M HCl, and absorbance readings (450–540 nm) were taken using an ELISA reader.

**Statistical Methods.** The significance analysis of the differences observed between different groups regarding serum PSA levels, primary lung tumor burden, and metastasis to liver and the adrenal gland was determined by using the standard Student’s t test analysis. The significance of the differences between the groups in the survival experiment was determined using the Mantel-Cox log-rank test.

**RESULTS**

**Anti-Human β₁, Fab’ Immunoliposomes Bind to and Are Internalized Efficiently by Human Lung Carcinoma Cells in Vitro.** To be effective as a drug-delivery system, immunoliposomes must bind selectively to the tumor target and be internalized into the tumor cells. The tumor target being tested here is the β₁ integrin family, which is expressed on the majority of human non-small cell lung carcinomas. A monoclonal antibody (1F11) specific for the common β chain of this integrin family was generated, and 1F11 Fab’ fragments of the antibody were conjugated to sterically stabilized liposomes (i.e., Fab’ anti-β₁ immunoliposomes). To determine whether these immunoliposomes bound selectively to the β₁ integrin target, a β₁ integrin-positive human lung tumor cell line RPCI-2E9 was incubated with immunoliposomes loaded with a fluorescent marker (i.e., Texas Red). After incubation at 4°C, the bound liposomes were removed by pelleting the tumor cells, and the resuspended cells were analyzed by confocal microscopy. Immunoliposomes (Fig. 1C), but not control liposomes (Fig. 1, A and E; i.e., liposomes without antibody or liposomes conjugated with an isotype control antibody 2C3), bound to the cell surface of the RPCI-2E9 tumor cells. Fluorescent immunoliposomes were diffusely distributed over the entire surface of the tumor cells. To determine whether the bound liposomes were internalized, the cells were incubated at 37°C for 1 h and reexamined. Confocal microscopy established that the bound liposomes were efficiently internalized as evidenced by the large aggregates of red fluorescence observed within the cell (Fig. 1D). No fluorescent ag-
gregates were observed in the tumor cells pulsed with control liposomes and incubated at 37°C (Fig. 1, B and F). These studies establish that the β1 integrin on human lung tumors represents experimentally a viable target for testing the ability of immunoliposomes to deliver cytotoxic drugs to a human solid tumor.

**Anti-β1 Fab’ Immunoliposomes Loaded with Doxorubicin Are Superior to Other Liposome/Doxorubicin Formulations in Inducing Tumor Cell Death in Vitro.** To determine whether the binding and internalization of immunoliposomes by tumor cells resulted in enhanced delivery of cytotoxic drugs to cells in vitro, anti-β1 Fab’ fragments were conjugated to sterically stabilized liposomes loaded with doxorubicin, and the ability of these immunoliposomes to induce cell death was compared with free doxorubicin and control liposome/drug formulations in an in vitro cytotoxicity assay. The results shown in Fig. 2A demonstrate that the anti-β1 Fab’-doxorubicin-loaded liposomes (1F11-s-dox) were 30-fold more effective than no-antibody/doxorubicin-loaded liposomes (s-dox) or than control isotype-matched Fab’ doxorubicin-loaded liposomes (2C3-s-dox). Interestingly, 1F11-s-dox liposomes were also 3-fold more effective in inducing tumor cell death than free doxorubicin in vitro. Anti-β1 Fab’ liposomes without doxorubicin (1F11-s) were not cytotoxic to cells at doses where >95% of the cells were killed by 1F11-s-dox, indicating that the observed tumor killing was induced by intracellular delivery of doxorubicin and was not simply attributable to the toxicity of internalized lipid (Fig. 2B).

The specificity of tumor cell killing by 1F11-s-dox liposomes was evaluated in a competitive inhibition binding study. Increasing amounts of free 1F11 antibody were added to the cell cultures along with a cytotoxic amount of 1F11-s-dox to determine whether competitive binding by free antibody could suppress the 1F11-s-dox induced cell death. The results are shown in Fig. 2C. Incubation of tumor cells with 1F11-s-dox liposomes induced complete tumor cell death, whereas the addition of increasing amounts of free 1F11 antibody reduced cytotoxicity of 1F11-s-dox in direct proportion to the antibody concentration. Incubation of tumor cells with a nonspecific, isotype-matched antibody (2C3) or with 1F11 did not affect cell viability significantly, except that a slight (~10%) but consistent suppression of cell growth was observed with 1F11. The results demonstrate that cytotoxicity was dependent on the specific binding of the 1F11 Fab’ to the β1 integrin on the surface of the cells.

**Engraftment of Human Lung Tumor into Lungs of SCID Mice.** To test the ability of immunoliposomes to deliver doxorubicin to human tumors in vivo, an orthotopic tumor xenograft model was established in SCID mice. The RPCI-2E9 cell line was inoculated i.v. into SCID mice, and tumor nodules forming in the lungs of SCID mice were removed and reinoculated into another set of SCID mice. This procedure was repeated four times, thereby establishing a tumor subline adapted to engraft and grow well orthotopically in the lungs of SCID mice (RPCI-2E9/IV4). Next, the tumor subline was transfected with a mammalian expression vector containing the gene encoding human PSA. The stably transfected tumor cell line RPCI-2E9/IV4/PSA, when injected i.v. into SCID mice, engrafts within the lung and releases human PSA into the serum of tumor-bearing mice. Thus, PSA in the serum serves as an indicator of the tumor engraftment, and increasing serum PSA levels were shown to correlate with tumor progression (30). This model was used here to evaluate the ability of the anti-β1 immunospecific liposomes to target the delivery of doxorubicin.
rubicin to the \( \beta_1 \)-positive human lung tumor and to compare its therapeutic efficacy to control liposomes and to free drug.

**Effect of Doxorubicin-loaded Anti-\( \beta_1 \) Fab’ Immunoliposomes on the Growth of Orthotopically Established Human Lung Tumor Xenografts in SCID Mice.** SCID mice were inoculated i.v. with RPCI-2E9/IV4/PSA tumor cells. The mice remained untreated for 1 week to allow tumors to establish within the lung. One week after tumor inoculation, mice received weekly i.v. injections of 1F11-Fab-dox-immunoliposomes, or control liposome formulations for a total of six treatments. Another group of mice received no treatment. Mice were bled weekly between weeks 4 and 7 after tumor inoculation, and tumor growth was estimated by quantifying serum PSA levels periodically by ELISA. PSA was detectable in the sera of untreated mice by week 4 and mice treated with control liposome formulations by week 5. PSA was not detected in mice treated with immunospecific liposomes until weeks 6 or 7 after tumor inoculation (data not shown). In three independent experiments, serum PSA levels of tumor-bearing mice treated with immunospecific liposomes were substantially lower than in mice receiving no treatment (Fig. 3). The immunospecific liposomes, i.e., 1F11-s-dox were also shown to be more effective than free doxorubicin, i.e., f-dox (Fig. 3A) or doxorubicin in control liposomes (drug-loaded liposomes without antibody), i.e., s-dox (Fig. 3, A and C), drug-loaded liposomes with an isotype control antibody, i.e., 2C3-s-dox (Fig. 3) or immunospecific liposomes without doxorubicin, i.e., 1F11-s (Fig. 3B). These results establish that in all three experiments tumor growth was suppressed more effectively in mice treated with the 1F11-s-dox immunoliposomes as compared with mice treated with free drug, the control liposome treated mice or untreated mice.

**Treatment of SCID Mice Bearing Lung Tumor Xenografts with Immunoliposomes Enhances Survival.** Although the results presented in Fig. 3 clearly establish the superiority of immunoliposomes in suppressing the growth of established tumor, evidence of some tumor growth (i.e., serum PSA levels) was observed in the immunoliposome-treated groups in two of the three experiments. Another

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**Fig. 2. In vitro cytotoxicity of immunoliposome formulations.** Tumor cells were incubated with the indicated amount of liposomes for 5 days in culture, after which cell numbers were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Inhibition of cell growth was determined and reported as percentage of control cell growth inhibition for cells that were incubated in culture medium alone. A, comparison of free drug (f-dox), no-antibody, doxorubicin-loaded liposomes (s-dox), control doxorubicin-loaded immunoliposomes (2C3-s-dox), and tumor-specific doxorubicin-loaded immunoliposomes (1F11-s-dox). B, comparison of empty (1F11-s) and doxorubicin-loaded (1F11-s-dox) tumor-specific immunoliposomes. C, the specificity of tumor cell killing by immunoliposomes. Tumor cells were incubated with 1F11 immunoliposomes (0.5 \( \mu \)g of doxorubicin) in the presence of increasing amounts of control (2C3) or blocking (1F11) antibody. The effect of free antibody on tumor cell number was also determined by incubation of cells with free antibody alone. Each value is an average of triplicate wells; bars, SD.
common indicator of efficacy that is used is enhanced survival of tumor-inoculated mice. To investigate the effect of different treatments on survival, mice were treated 1 week after tumor inoculation and monitored for survival for up to 19 weeks. All treated groups received six weekly treatments. Mice experiencing one or more of the following clinical signs were sacrificed: paralysis, dyspnea, or cachexia. By 12 weeks, all untreated tumor-bearing mice had died or were sacrificed. Treatment of mice with free doxorubicin actually decreased the survival time at the dose and schedule used (all mice were dead by week 10), possibly because of a combination of tumor growth and toxicity of the drug (Fig. 4). The median survival time of the mice that received doxorubicin-loaded control liposomes (either no antibody s-dox or control antibody 2C3-s-dox) was similar to mice that received no treatment (all mice were dead by week 12 or 13). The median survival time of mice treated with the immunoliposomes (1F11-s-dox) was increased ~35%, with 80% of the mice still alive between weeks 15 and 16. We conclude that the immunoliposomes compared with either free drug or control liposomes preparations increase survival time of tumor-bearing mice and are therefore more efficient at delivering a cytotoxic drug to a solid tumor in vivo.

Immunospecific Liposomes Prevent Metastasis of Human Lung Tumor Xenografts in SCID Mice. All mice inoculated i.v. with RPCI-2E9/IV4/PSA have tumors initially established in the lung. Tumors are observed macroscopically in the lungs 1 week after inoculation, and by 4–5 weeks, lung tumor nodules can be seen macroscopically (data not shown). By 6–8 weeks, metastatic tumor nodules appear in the liver and adrenal glands of tumor-inoculated mice. To assess the effect of different treatments on tumor metastasis, mice were inoculated with RPCI-2E9/IV4/PSA cells and treated with either immunospecific liposomes (1F11-s-dox) or control liposomes (s-dox or 2C3-s-dox) six times at weekly intervals 1 week after tumor inoculation. All mice were sacrificed 8 weeks after tumor inoculation; the number of tumor nodules in the lungs was counted, and the degree of metastasis to the liver and adrenal glands was estimated by weighing these organs. Results of the treated groups were compared with untreated tumor-inoculated mice. A summary of two experiments is presented in Fig. 5. In both experiments, the number of tumor nodules in the lung was suppressed in the 1F11-s-dox-treated group compared with the no-treatment group. In one experiment (Fig. 5A), there was no statistically significant difference in the total number of lung tumor nodules in the s-dox- and 1F11-s-dox treated groups. However, the tumor nodules in the 1F11-s-dox-treated group were smaller, and 1F11-s-dox completely blocked metastasis of the tumor to the adrenal gland and dramatically suppressed metastasis to the liver, whereas control treatment groups had little or no consistent effect upon metastasis to either the liver or adrenal glands (Fig. 5, C–F). These results establish in this tumor model that an additional therapeutic advantage of the immunospecific liposomes over the nontargeted liposomes is the suppression or prevention of the metastatic spread of the tumor.

DISCUSSION

The data presented here establish that sterically stabilized immunoliposomes can be selectively targeted to and have preferential cytotoxicity for human lung tumor xenografts established orthotopically in the lung of SCID mice. Treatment of mice with long-circulating immunospecific liposomes suppresses the growth of tumors, resulting in increased mean survival times compared with mice treated with nontargeted liposomes or free drug. Suppression of pre-existing tumors indicates the ability of the immunoliposomes to extravasate, home to, and kill the primary tumor in the lung.

In this study what is particularly interesting and potentially clinically relevant is the suppression of tumor metastases from the lung to liver and adrenal glands observed in lung tumor-bearing mice treated with the 1F11-s-dox (immunospecific) liposomes. Although there was some suppression of primary tumors observed with the nontargeted liposomes, no consistent suppression of tumor metastases was observed in tumor-bearing mice treated with these control liposomes. These results suggest that the optimal clinical use of the antibody-targeted liposomes may be in conjunction with surgery to suppress smaller tumors in the lung and especially to suppress or prevent metastatic disease.

The in vitro and in vivo efficacy of sterically stabilized immunoliposomes and their increased effectiveness when prepared using intact antibodies (but not Fab’ fragments) has been reported previously using murine tumor models (33–35). However, little very information exists on the utility of these immunospecific liposomes in the treatment of human cancer. In the one previous in vivo study of human tumors, immunoliposomes were shown to be superior to control nontargeted liposomes in suppressing the growth of a human B-cell lymphoma xenograft in SCID mice (36). In this study, sterically stabilized liposomes were targeted to a differentiation antigen (CD19) expressed on the tumor. Mice were inoculated with the tumor either i.p. or i.v. and were treated with immunoliposomes injected i.p. or i.v. 1 or 24 h after tumor inoculation. The immunoliposomes loaded with doxorubicin were shown to significantly increase the survival time compared with tumor-inoculated mice receiving either free drug or drug-loaded, nontargeted liposomes. Although our study confirms the efficacy of immunoliposomes in vivo with another human tumor and a different tumor target, it differs in several other significant ways from the initial report with B-cell lymphomas. In the present study, treatment was not started until 7 days after tumor inoculation, and the human lung tumor xenografts were established orthotopically, i.e., in the lung of SCID mice. The design of this study also made it possible to monitor tumor growth continuously by assaying the serum of tumor-bearing mice for a tumor marker, thereby providing a quantifiable indicator of tumor progression for establishing the efficacy of the liposome treatment that is independent of survival times. In contrast to the B-cell lymphoma study, we have used Fab’ fragments of the antibody in preparing the immunoliposomes. This has been shown previously to enhance the circulation time and enhance the extravasation of immunoliposomes into tumors in a mouse tumor model (37). Another significant, and perhaps the most important, difference between the two models is that we were able to observe in the lung tumor xenograft model the metastatic spread of the lung.
tumors to the liver and adrenal, making it possible to establish another beneficial effect of the immunospecific liposomes, i.e., suppression of tumor metastases.

It is important to emphasize that in neither the work reported here nor in the one previous study with a human tumor (36) was it possible to completely eradicate established human tumors with the dose and schedules of immunospecific liposomes used. We have tried to eradicate tumors without success using a single high dose of or additional low doses of the drug-loaded immunoliposomes. For example, we have compared the effect of a single high dose of doxorubicin (3 mg/kg) delivered in immunospecific liposomes to six low doses of doxorubicin (0.5 mg/kg) delivered in immunospecific liposomes on the growth and metastasis of the RPC-2E9/PSA-transfected lung tumor in SCID mice. Eight weeks after tumor engraftment and 7 weeks after the initial treatment, we saw no significant difference in the serum levels of PSA (i.e., 1.9 ± 1.1 ng/ml for multiple dose and 1.4 ± 1.8 ng/ml for single dose, P = 0.548) and only a modest difference in the number of tumor nodules in the lung, i.e., 13 ± 4 in mice treated with multiple low doses of doxorubicin versus 18.0 ± 6.5 for the mice treated once with a high dose of the drug, P = 0.195. The mean tumor weights in the liver of mice receiving multiple doses of the drug-loaded immunoliposomes was 13 ± 21 mg and 45 ± 33 mg in mice treated with a single dose, P = 0.105. Thus, there appears to be no significant potential therapeutic benefit of giving multiple low doses to a single high dose of the drug in the immunospecific liposomes. These results are consistent with the results in previous studies in lymphoma and lung cancer (35, 36), which established that most of the therapeutic effect of the immunoliposomal doxorubicin is associated with the first injection, with subsequent injections having little additional effect. Thus, although immunoliposomes represent an improved drug delivery system, it is apparent that they still have limitations. The inability of immunoliposomes to completely eradicate established tumors has been recognized previously and suggested to be attributable, at least in part, to the inability of the liposomes to penetrate into the interior of existing solid tumor nodules (38, 39). Thus, the use of targeted liposomes alone may not be an effective treatment for cancer, but they may be useful when used in conjunction with other therapeutic modalities including surgery, radiation, or immunotherapy.

This study has also provided some important insights with respect to the potential and the limitations of SCID mice as a model in which to study and evaluate anticancer therapeutic modalities. Since the first successful xenograft of a human tumor in SCID mice was reported (40), the SCID mouse has become a popular host for growing human neoplasms and has been used effectively to evaluate different therapeutic strategies (8–10). However, the observation made here that free
doxorubicin treatment of tumor-bearing SCID mice actually decreased survival compared with untreated mice (Fig. 4) reflects one of the limitations of the SCID model. SCID mice have a defect in repair of double-stranded breaks in DNA such as those mediated by free oxygen radicals (41, 42). Although the exact mechanism of the antitumor activity of doxorubicin remains unclear, some of its associated toxicities have been attributed to the formation of free radicals (43), which would make this drug even more toxic in SCID mice.

We have observed previously enhanced toxicity of doxorubicin in SCID mice (8) and have been able to alleviate some of the toxicity by scheduling multiple injections at lower doses, similar to what is observed in humans with doxorubicin (44). We have used this strategy in the work presented here. One of the distinct advantages of the SCID model is the ability of human tumors to be engrafted orthotopically and to metastasize in SCID mice as is demonstrated here. Another advantage of SCID mice is based upon previous studies that have established that human immunocompetent cells can be engrafted into SCID mice (45) and that these cells remain functional (46). Coengraftment of peripheral blood lymphocytes (47) or tumor-infiltrating lymphocytes (48) into SCID mice have shown that these cells are able to generate an antitumor response that can be augmented with cytokine immunotherapy. It is apparent that the SCID mouse would provide a viable model in which to evaluate a combined cancer therapeutic approach in which tumor-bearing mice coengrafted with peripheral blood leukocytes are treated with immunoliposomes initially followed by immunotherapy with cytokines.

The β1 integrin in these studies has proven to be a viable target for establishing the proof of principle for immunoliposome delivery to a human lung tumor in our experimental model. The Fab' fragments of antibodies were shown to be effective in directing the selective binding of the liposomes to a β1 integrin expressing human lung tumor in vitro, and more importantly, the bound liposomes are shown to be subsequently internalized by the tumor. Whether this particular family of heterobifunctional adhesion molecules turns out to be a suitable target to be used in a clinical setting has not been addressed here.

In this regard, it is of interest to note that many different tumors have been shown to up-regulate their expression of one or more of the members of the β1 integrin family. For example, αvβ3 integrins are overexpressed in some invasive bladder carcinomas but not in normal bladder epithelium (49). Increased levels of the αvβ1 integrin have been found on a number of different cancers including non-small cell lung carcinomas (50), pancreatic carcinomas (51), and invasive melanoma (52, 53). However, these integrins also have a relatively wide distribution pattern on many normal tissues including epithelium and some leukocytes that may limit their usefulness as a target for liposome delivery in a clinical setting. The αvβ1 integrins in human lung tumors (27). Preferential binding of anti-integrin-targeted liposomes to tumors in the lung may be further enhanced by the natural homing tendency of liposomes to the lung and binding to integrin expressing normal tissue could possibly be avoided further by injecting liposomes directly into the lung via the pulmonary artery. Although we have established the feasibility and potential of targeting liposomes to lung tumors experimentally, additional preclinical studies are necessary to assess the real clinical utility and safety of using one or more β1 integrins as targets for the delivery of drug-loaded liposomes to tumors.

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