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Efficient and Sustained Gene Expression in Primary T Lymphocytes and Primary and Cultured Tumor Cells Mediated by Adeno-Associated Virus Plasmid DNA Complexed to Cationic Liposomes

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We have used cationic liposomes to facilitate adeno-associated virus (AAV) plasmid transfections of primary and cultured cell types. AAV plasmid DNA complexed with liposomes showed levels of expression several fold higher than those of complexes with standard plasmids. In addition, long-term expression (>30 days) of the gene, unlike the transient expression demonstrated by typical liposome-mediated transfection with standard plasmids, was observed. Southern analysis of chromosomal DNA further substantiated the hypothesis that the long-term expression was due to the presence of the transgene in the AAV plasmid-transfected group and not in the standard plasmid-transfected group. AAV plasmid-liposome complexes induced levels of transgene expression comparable to those obtained by recombinant AAV transduction. Primary breast, ovarian, and lung tumor cells were transfectable with the AAV plasmid DNA-liposome complexes. Transfected primary and cultured tumor cells were able to express transgene product even after lethal irradiation. High-level gene expression was also observed in freshly isolated $CD3^+$, $CD4^+$, and $CD8^+$ T cells from normal human peripheral blood. Transfection efficiency ranged from 10 to 50% as assessed by intracellular interleukin-2 levels in interleukin-2-transfected cells. The ability to express transgenes in primary tumor and lymphoid cells may be applied toward tumor vaccine studies and protocols which may eventually permit highly specific modulation of the cellular immune response in cancer and AIDS.

Transfection of eukaryotic cells has become an increasingly important technique for the study and development of gene therapy. Advances in gene therapy depend in large part upon the development of delivery systems capable of efficiently introducing DNA into the target cell. A number of methods have been developed for the stable or transient expression of heterologous genes in cultured cell types. These include the use of carrier molecules or viruses.

Although extensive progress has been made, these techniques suffer from variable transfection efficiency, significant concern about possible recombination with endogenous virus, cellular toxicity, and immunologic host response reactions. These concerns have prompted the search for nonviral DNA transfection conditions for many cell types. Although nonviral techniques have overcome some of the problems of the viral systems, there remains a need for improved transfection efficiency in these newer nonviral systems (7, 13), although improved efficiency can be variably attained by the promoter enhancer elements utilized in the plasmid DNA constructs (19).

As a nonviral system, liposomes have been used to encapsulate and deliver to cells a variety of materials, including nucleic acids (3–5) and viral particles (6, 7, 18, 29). Recently, positively charged liposomes containing the membrane fusion-promoting lipid (*N*-[1,2,3-dioleyloxy]propyl)-N_1,N_1,*N*-triethylammonium have been shown to efficiently transfer heterologous genes into eukaryotic cells (7, 20). Cationic liposomes spontaneously complex with plasmid DNA or RNA in solution and facilitate fusion of the complex with cells in culture, resulting in the efficient transfer of nucleic acids to a wide variety of eukaryotic cell types.

Of the viral vector systems, the recombinant adeno-associated virus (AAV) transduction system has proven to be one of the most efficient vector systems to stably carry the genes with high efficiency into a variety of mammalian cell types (17). AAV is a linear single-stranded DNA parvovirus which is dependent upon coinfection by a second unrelated virus to undergo productive infection. AAV carries two sets of functional genes: the *rep* genes, which are necessary for viral replication, and the structural capsid protein genes (10, 27). The *rep* and capsid genes of AAV can be replaced by a desired DNA fragment to generate AAV plasmid DNA. Transcomplementation of *rep* and capsid genes is required to create a recombinant virus stock. Upon infection, the recombinant virus uncoats in the nucleus and integrates into the host genome by its molecular ends (16, 21). It has been well documented that AAV DNA integrates into cellular DNA as one to several tandem copies joined to cellular DNA through the termini (1, 14, 23).

The AAV terminal repeats are an essential part of this transduction system, and recombinant virus is made primarily to transport the AAV genome into the cells. We examined whether AAV plasmid DNA transported into the cells by a nonviral system could be used to create systems that efficiently transfect primary mammalian cell types. The system developed here takes advantage of the simple carrier system of lipofection and the proficient expression capability of the AAV plasmid construct.

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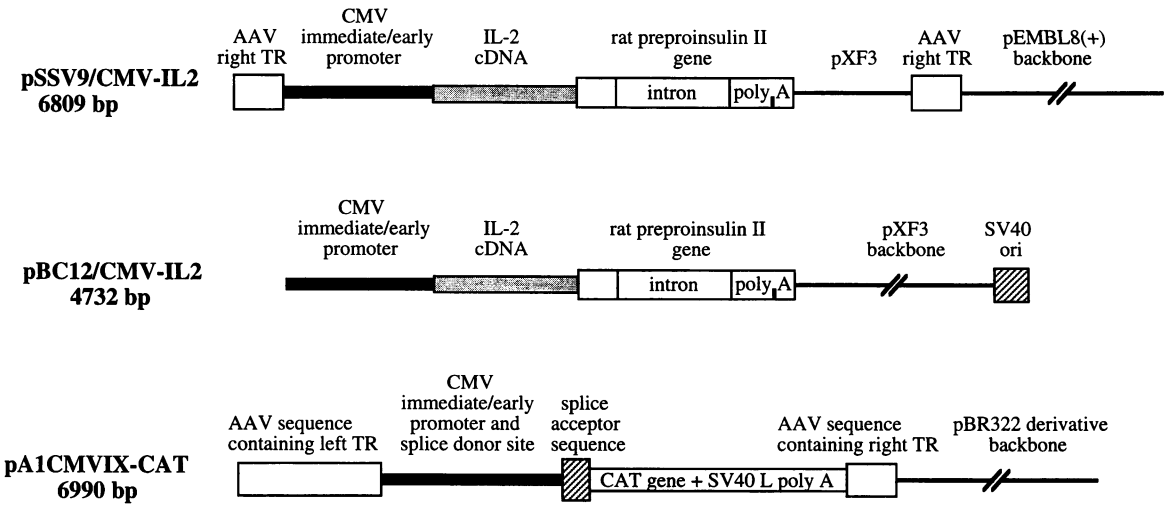

FIG. 1. Plasmid maps. Plasmid pSSV9/CMV-IL2 contains the CMV promoter, IL-2 cDNA, and rat preproinsulin and simian virus 40 (SV40) polyadenylation sequences identical to those in the pBC12/CMV-IL2 plasmid, along with AAV ITRs at both ends. Plasmid pA1CMVIX-CAT was constructed with the CMV promoter and CAT gene inserted between the two AAV ITRs.

In this study we examined the utility of the cationic liposomes as a means to facilitate the AAV plasmid DNA entry into the cells in the absence of *rep* and capsid transcomplementation, recombinant virus, or wild-type AAV. In addition, we explored the utility of liposomes to replace the AAV-mediated transduction yet maintain a high efficiency of gene expression. The data indicate that AAV cationic liposome gene transfer can be used to efficiently transfect primary lymphoid cells, a variety of freshly isolated tumor cells, and cultured mammalian cell types with sustained expression of DNA.

MATERIALS AND METHODS

Cell lines. The rat prostate cell line R3327 and bladder cell line MBT-2 were obtained from Eli Gilboa, Duke University. Both cell lines were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS). Cell line 293 is a human embryonic kidney cell line that was transformed by adenovirus type 5 (9). This cell line was grown in Dulbecco modified Eagle medium supplemented with 10% FBS.

Cell preparation. Primary lung, ovarian, and breast tumor cells were obtained from the solid tumors of patients. The tumor samples were minced into small pieces and digested in 200 ml of AIM V medium (GIBCO, Grand Island, N.Y.) supplemented with 450 U of collagenase IV (Sigma, St. Louis, Mo.) per ml, 10.8 Klett units of DNase I (Sigma) per ml, and 2,000 U of hyaluronidase V (Sigma) per ml (26). After 1 to 2 h of digestion, cells were homogenized with a glass homogenizer (Bellco, Vineland, N.J.). Cells were washed three times in DPBS-CMF (Whittaker, Walkersville, Md.). Lymphocytes were separated from nonlymphoid cells by capture on a MicroCELLector-CD5/8 device (Applied Immune Sciences [AIS], Santa Clara, Calif.). The MicroCELLectors are polystyrene devices containing covalently immobilized monoclonal antibodies for selection of T cells. Nonadherent cells (mainly tumor cells) were removed and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U of penicillin-streptomycin per ml, and 10% FBS. Tumor cells were cultured for 2 to 4 weeks prior to transfection.

Peripheral blood mononuclear cells from healthy controls were isolated from buffy coats (Stanford University Blood Bank, Stanford, Calif.) by using Lymphoprep (Robbins Scientific, Sunnyvale, Calif.). T cells or T-cell subsets were further isolated with AIS MicroCELLectors. Briefly, peripheral blood mononuclear cells were resuspended at 15×10^6 cells per ml in 0.5% Gamimmune (Miles, Inc., Elkhart, Ind.) and loaded onto the washed CD3, CD4, or CD8 AIS MicroCELLectors. After 1 h, nonadherent cells were removed. Complete medium (RPMI 1640 medium [Whittaker] containing 10% FBS, 2 mM L-glutamine, and 100 U of penicillin-streptomycin per ml) was added to the adherent cells in the MicroCELLectors. After 2 to 3 days in a 5% CO_2, 37°C humidified environment, adherent cells were removed and prepared for transfection.

Plasmid preparation. We used a plasmid containing the human interleukin-2 (IL-2) gene and the immediate-early promoter-enhancer element of the human cytomegalovirus (CMV) flanked by AAV terminal repeats, pSSV9/CMV-IL2 (Fig. 1), provided by J. Rosenblatt (University of California, Los Angeles), and the IL-2 plasmid without AAV terminal repeats, pBC12/CMV-IL2 (Fig. 1), was obtained from American Type Culture Collection. Plasmid pA1CMVIX-CAT contains the CMV immediate-early promoter enhancer sequences and some intervening sequences with splice acceptor sequences derived from an immunoglobulin G variable region (pOG44; Strategene, La Jolla, Calif.), the bacterial chloramphenicol acetyltransferase (CAT) gene, and the simian virus 40 late polyadenylation signal flanked by AAV terminal repeats in a pBR322 derivative (Fig. 1). Plasmids were isolated by alkaline lysis and ammonium acetate precipitation followed by treatment with DNase-free RNase, phenol-chloroform-isoamyl extractions, and ammonium acetate precipitation (2).

Liposome preparation. Small unilamellar liposomes were prepared from the cationic lipid dimethyldioctadecylammonium bromide (DDAB) (Sigma) in combination with the neutral lipid dioleoylphosphatidylethanolamine (DOPE) or cholesterol (Avanti Polar Lipids, Alabaster, Ala.). Lipids were dissolved in chloroform. DDAB was mixed with DOPE or cholesterol in either a 1:1 or 1:2 molar ratio in a round-bottomed flask, and the lipid mixture was dried on a rotary evaporator. The lipid film was rehydrated by adding sterile double-distilled water to yield a final concentration of 1 mM

DDAB. This solution was sonicated in a bath sonicator (Laboratory Supplies, Hicksville, N.Y.) until clear. Liposomes were stored at 4°C under argon.

Preparation of rAAV stocks. For the preparation of recombinant AAV (rAAV) stocks, 293 cells were split and grown to approximately 30 to 50% confluence. At this time, the cells were infected with adenovirus type 5 and incubated at 37°C. After 2 to 4 h, the infected cells were cotransfected with 10 μg of plasmid and 10 μg of the *rep* capsid complementation plasmid, pΔBal, per 100-mm-diameter tissue culture dish (0.5×10^7 to 1×10^7 cells). Calcium phosphate coprecipitation was used for transfection (11). At 12 to 18 h after transfection, the medium was removed from the cells and replaced with 5 ml of Dulbecco modified Eagle medium containing 10% FBS. At 48 to 72 h posttransfection, AAV was harvested as follows. The cells and medium were collected together and freeze-thawed three times to lyse the cells. The medium-cell suspension was then centrifuged to remove cellular debris, and the supernatant was incubated at 56°C for 1 h to inactivate adenovirus (11, 28). After heat inactivation, the viral supernatant was filtered through cellulose acetate filters (1.2-μm pore size). Viral stocks were then stored at −20°C. rAAV stock containing a 10^4 viral titer was used to infect cells.

Cellular transfection. For primary tumor cells and tumor cell lines, 10^6 cells were plated in 2 ml of serum-free medium per well of a six-well dish. Plasmid DNA (10 μg) was mixed with 30 nmol of total lipid as liposomes composed of DDAB and DOPE in a 1:2 molar ratio. Serum-free medium (0.5 ml) was added to the liposome-DNA complex, which was then transferred to the cells. The cells were incubated at room temperature for 5 min, and then fetal calf serum was added to the cells to yield a final concentration of 5% fetal calf serum. For T cells, 5×10^6 to 10×10^6 cells were plated in 1 ml of serum-free medium per well of a six-well dish. Plasmid DNA (50 μg) was mixed with 100 nmol of total lipid as liposomes composed of DDAB and DOPE or cholesterol in a 1:1 molar ratio. The transfections were then performed as above.

IL-2 assay. Cells were counted, 10^5 cells were plated in 1 ml per well of a 24-well plate. The following day, supernatants were collected and assessed by using a Quantikine IL-2 enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, Min.). IL-2 levels are expressed as picograms per milliliter of the supernatant.

Intracellular IL-2 assay. Transfected cells were stained at various time points for intracellular IL-2 protein levels by a modified flow cytometry procedure (15). Cells were harvested, washed with DPBS-CMF (Whittaker) and resuspended at 10^6 cells per ml in cold 1% paraformaldehyde (Sigma) in DPBS-CMF for 10 min at 4°C. Cells were washed with DPBS-CMF and resuspended in cold DPBS-CMF containing 0.1% saponin (Sigma) and 10% FBS (HyClone, Logan, Utah) for 10 min at 4°C. Cells were then washed with cold saponin buffer and stained with mouse anti-human IL-2 antibody (Genzyme, Cambridge, Mass.) for 15 min at 4°C. Cells were washed with cold saponin buffer and stained with a fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ second-step antibody (Caltag, South San Francisco, Calif.) for 15 min at 4°C. After washing in saponin buffer, cells were washed with DPBS-CMF and resuspended at 10^6 cells per ml for flow cytometric analysis. Flow cytometry was performed with a FACScan (Becton Dickinson, Milpitas, Calif.).

CAT assay. To measure CAT activity, the transfected cells were collected, washed twice with phosphate-buffered saline, and then resuspended in 0.25 M Tris, pH 7.8. Cell extracts were obtained by three consecutive freeze-thaw cycles followed by centrifugation at 16,000 × *g* for 5 min. Protein concentrations of extracts were measured by a Coomassie blue G250-based assay (Bio-Rad, Richmond, Calif.), and protein concentrations were normalized. A volume of extract was added to 200 nmol of acetyl coenzyme A and either 0.1 (R3327 rat prostate cells) or 0.5 (T cells) μCi of [^{14}C]chloramphenicol (Amersham, Arlington Heights, Ill.). The reaction mixture was incubated at 37°C for 16 h. The acetylated and unacetylated chloramphenicol species were extracted with cold ethyl acetate and resolved on silica thin-layer chromatography plates with 95:5 (vol/vol) chloroform-methanol solvent. The radiolabeled products were visualized by autoradiography.

Southern blot analysis of chromosomal DNA. For Southern analysis, chromosomal DNA was extracted from cells by the procedure described by Hirt (12). After digestion with appropriate restriction enzymes, 5 μg of DNA was loaded onto a 1% agarose gel, electrophoresed, and transferred to Hybond N+ (Amersham) nylon membrane. The membranes were hybridized with a 0.685-kb IL-2 gene fragment at 65°C in rapid hybridization buffer with DNA fragments labeled with ^{32}P by the random priming method (Megaprime DNA labeling kit; Amersham) and washed according to the manufacturer's instructions. Autoradiograms of these filters were exposed on X-ray film (type XAR; Eastman Kodak Co.) with intensifying screens at −70°C for 1 to 4 days.

RESULTS

AAV plasmid-cationic liposome complexes induce high-level gene expression. To evaluate the gene transfer efficiency of AAV plasmids, the IL-2 gene transfer efficiency of AAV plasmids was compared with the efficiency of standard plasmids. The standard plasmid constructs carrying the IL-2 gene were driven by the adenosine deaminase promoter, a thymidine kinase promoter, or a CMV promoter. The AAV plasmid (pSSV9/CMV-IL2) contained the CMV immediate-early promoter with the IL-2 gene placed downstream of the promoter (Fig. 1). As shown in Fig. 1, the corresponding control plasmid, the pBC12/CMV-IL2 construct, was identical to pSSV9/CMV-IL2 but lacked the AAV inverted terminal repeats (ITRs). All five plasmids containing the IL-2 gene were complexed with liposomes and tested for transfection efficiency on the two cultured tumor cell lines: the rat bladder (MBT-2) and the rat prostate (R3327) cell lines. The cell lines were transfected with 10 μg of plasmid DNA complexed to 30 nmol of liposomes per 10^6 cells. Supernatants were collected on day 3 and tested for levels of IL-2 with an IL-2 ELISA kit.

AAV plasmid pSSV9/CMV-IL2 induced the highest levels of expression in both cell lines (Fig. 2a). The IL-2 gene with an adenosine deaminase promoter induced the least amount of expression in both cell lines. As shown in Fig. 2a, both thymidine kinase promoter–and CMV (immediate-late) promoter–IL-2 gene constructs induced comparable levels of IL-2 expression in both cell lines. However, the pBC12/CMV-IL2 plasmid, which contains the CMV immediate-early promoter, showed higher levels of gene expression in the prostate cell line than in the bladder cell line. Among the plasmids tested, the AAV IL-2 plasmid induced the highest level of expression in both cell lines, with significant level of increase observed in the prostate cell line.

As illustrated in Fig. 2b, the duration of expression induced by the corresponding control plasmid (pBC12/CMV-IL2) and the AAV IL-2 plasmid (pSSV9/CMV IL2) in the prostate cell line, R3327, was studied. The data in Fig. 2b indicate that in addition to the enhanced levels of expression, expression lasted for up to 30 days posttransfection with the AAV plasmid (pSSV9/CMV-IL-2). Both plasmids induced maximum levels

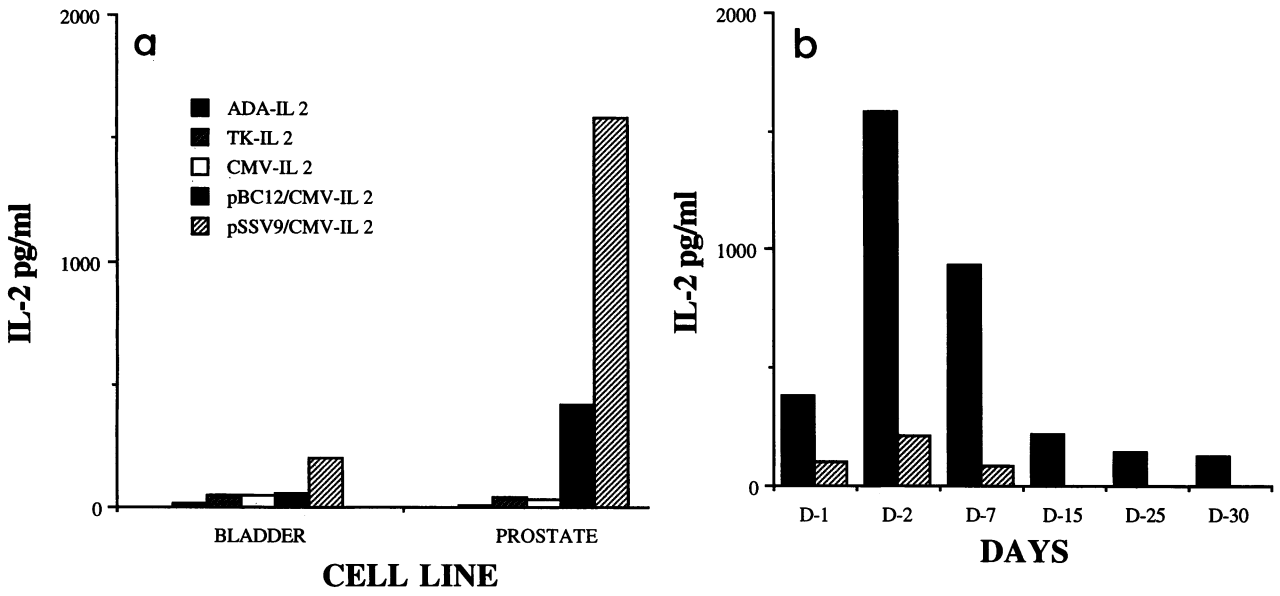

FIG. 2. (a) AAV plasmid DNA-liposome complexes induce high-level gene expression. Various IL-2 plasmid constructs were tested for their capability to induce gene expression in two cell lines when they were complexed with liposomes. In both cell lines, the AAV plasmid construct showed the highest level of expression. The cell lines were transfected with 10 μg of plasmid DNA complexed to 30 nmol of liposomes per 10^6 cells. Supernatants were collected on day 3 and tested for the levels of IL-2 with an IL-2 ELISA kit. The levels are expressed as picograms of IL-2 per milliliter per 10^6 cells in a 24-h culture period. ADA, adenosine deaminase; TK, thymidine kinase. (b) Long-term gene expression induced by AAV IL-2 plasmid-liposome complexes. To compare the duration of transgene expression, the prostate cell line was transfected with the standard plasmid (pBC12/CMV-IL2 [▨]) and the AAV plasmid (pSSV9/CMV-IL2 [■]) complexed with liposomes. Supernatants were collected at various time points and assayed for IL-2 levels by an ELISA. IL-2 levels are expressed per 10^6 cells in 24 h of culture.

of expression between days 2 and 7; by day 15 IL-2 levels declined and were maintained at approximately 100 pg/ml in the AAV plasmid-transfected group only. Similar sustained levels of expression were observed in the bladder cell line as well as with cells from a primary lung tumor, when AAV plasmid-liposome complexes were used for transfection (data not shown). In addition to the IL-2 plasmid, a CAT gene construct with a backbone and level of AAV ITR sequences different from those of the IL-2 construct was tested. The CAT gene construct produced similar sustained transgene expression (data not shown).

AAV plasmid transfection is comparable to rAAV transduction. The prostate and bladder cell lines were transfected and transduced to determine whether optimal AAV plasmid-liposome complex transfection was comparable to the optimal rAAV transduction. For optimal transfection, 10 μg of AAV plasmid DNA was complexed to 30 nmol of liposomes per 10^6 cells in a 2-ml final volume. For maximal rAAV transduction, 2 ml of the rAAV stock containing a 10^4 viral titer was added to 10^6 cells in 1 ml of complete medium.

In the prostate line, transfection induced higher levels of expression than AAV transduction under test conditions (Fig. 3a). Although results on days 3 through 5 showed approximately 10-fold higher levels of IL-2 with transfection, by day 20 comparable levels were observed in both transfected and transduced groups.

Transduction with rAAV initially induced higher levels of IL-2 production in the bladder cell line compared with the levels induced by transfection using liposomes (Fig. 3b). Similar to the prostate cell line, the bladder cell line also showed a decline in IL-2 levels by day 20, with comparable levels of IL-2 produced through day 33 in both transfected and transduced groups.

Primary tumor cells can be transfected with AAV plasmid DNA-liposome complexes. In the foregoing experiments, significant transgene expression was demonstrated in cultured cell lines. In order to assess whether cationic liposome-AAV plasmid DNA complexes also mediated comparable transgene expression in freshly isolated primary tumor cells, cells from four different primary tumors were transfected with the pSSV9/CMV-IL2 plasmid by using liposomes. Tumor cells were cultured in RPMI 1640 medium supplemented with 10% FBS for 2 to 3 weeks prior to the transfection. The cells were plated to a concentration of 10^6 cells per ml and transfected with 10 μg of DNA complexed with 30 nmol of liposomes. Supernatants were collected on days 2 and 3.

As shown in Fig. 4, all four primary cell types produced significant levels of IL-2 after transfection. The highest level of expression was observed on day 3. IL-2 gene expression was measured in cells from the lung tumor and in cells from one of the breast tumors for as long as 25 days posttransfection in culture (data not shown). Although the expression levels on day 3 were lower in the primary tumors than in the cell lines, the levels on day 15 were equivalent (100 pg of IL-2 per ml) in both cell lines and the cells derived from primary tumors (data not shown).

Lethal irradiation does not affect the transgene expression. To determine the effect of irradiation on gene expression, the prostate cell line (Fig. 5a) and cells of primary breast tumors (Fig. 5b) were transfected and assessed for gene expression after lethal irradiation. Both cell types were transfected with optimal AAV plasmid-liposome complexes. On day 2, an aliquot of the cells was subjected to 6,000 rads with a ^{60}Co irradiator, whereby cellular division was abolished, and the aliquots were kept in culture. One-half of each culture was maintained as a nonirradiated control. Supernatants were collected 24, 48, 72, and 96 h after irradiation from both

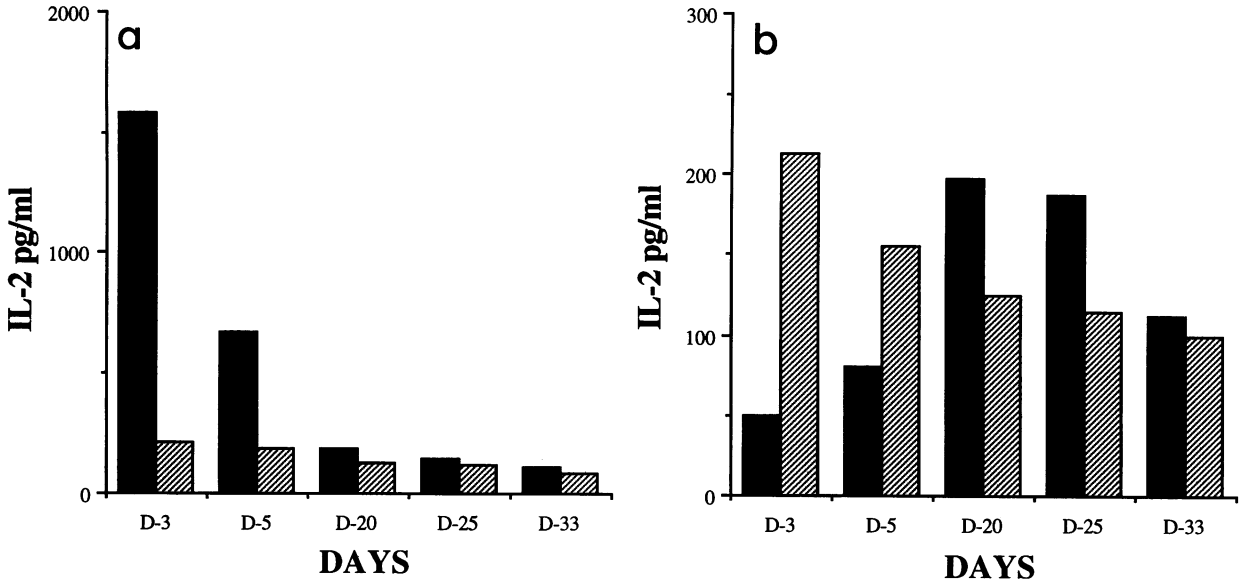

FIG. 3. Gene expression in AAV plasmid-liposome complex-mediated transfection compared with that in rAAV transduction. The prostate cell line (a) and bladder cell line (b) were used to study the transfection and transduction of the IL-2 gene. For optimal transfection, 10 μg of AAV plasmid DNA was complexed to 30 nmol of liposomes per 10^6 cells in a 2-ml final volume. For maximal rAAV transduction, 2 ml of the viral supernatant was added to 10^6 cells in 1 ml of complete medium. After 24 h, the cells were washed and resuspended in fresh complete medium. The supernatants were collected at various time points after transfection and transduction. IL-2 levels were assessed by an IL-2 ELISA. The levels are expressed as picograms per milliliter per 10^6 cells in 24 h of culture. ■, pSSV9/CMV-IL2; ▨, rAAV.

irradiated and nonirradiated cultures and tested for IL-2 levels.

As shown in Fig. 5, lethal irradiation posttransfection did not alter transgene expression. Neither the prostate cell line nor the primary tumor cells expressed any change in IL-2 expression after irradiation. Thus, although cellular division was abolished by irradiation, IL-2 expression and secretion were not sensitive to irradiation.

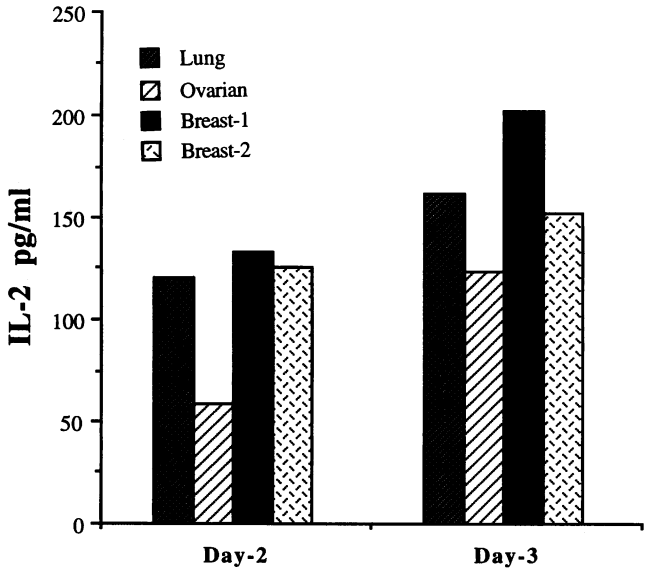

FIG. 4. AAV plasmid and liposome complexes transfect various primary tumor cells. One lung, one ovarian, and two breast tumor samples were isolated from fresh tumor biopsy specimens. Tumor cells were cultured in RPMI 1640 medium supplemented with 10% FBS for 2 to 3 weeks prior to the transfection. The cells were plated to a concentration of 10^6 cells per ml and transfected with 10 μg of DNA complexed with 20 nmol of liposomes. Supernatants were collected on days 2 and 3. IL-2 levels were measured by an ELISA. The levels are indicated as picograms per milliliter per 10^6 cells in 24 h of culture.

AAV plasmid transfection efficiency in a prostate cell line. To demonstrate the expression levels on a per-cell basis, the intracellular detection of IL-2 by flow cytometry assay was used to quantitate the percentage of cells expressing IL-2 protein. The prostate cell line was transfected with 10 μg of DNA complexed to 30 nmol of liposomes. At various time points, approximately 5×10^5 cells were harvested, fixed, permeabilized, stained with anti-IL-2 antibodies followed by fluorescently labeled second antibodies, and analyzed by flow cytometry.

Maximum transgene expression was observed between days 2 and 4 (Fig. 6). Significant levels of IL-2 expression were observed through day 22, with approximately one-fourth-maximal levels present on day 22. Flow cytometry analysis of IL-2-positive cells showed 10 to 50% transfection efficiency. The expression pattern and duration were similar to those of IL-2 protein secretion as assessed by ELISA as described above.

AAV plasmid-liposome complex induces transgene expression in a freshly isolated peripheral blood T-cell subpopulation. The capability of AAV plasmid-liposome complexes to transfect freshly isolated human peripheral blood T-cell populations was examined. The gene for CAT enzyme was used as the reporter gene in the pA1CMVIX-CAT plasmid (Fig. 1). Total and purified $CD4^+$ and $CD8^+$ subpopulations of T cells were used for transfections. CD3-, CD4-, or CD8-selected subpopulations of T cells showed significant levels of CAT gene expression (Fig. 7).

Primary T cells freshly isolated from peripheral blood were tested for transgene expression with AAV plasmid-liposome complexes. T lymphocytes were fractionated as $CD3^+$, $CD4^+$, or $CD8^+$ populations with AIS MicroCELLector devices. The relevant cells were captured, and nonadherent cells were washed off. The adherent cells were removed from the devices after 2

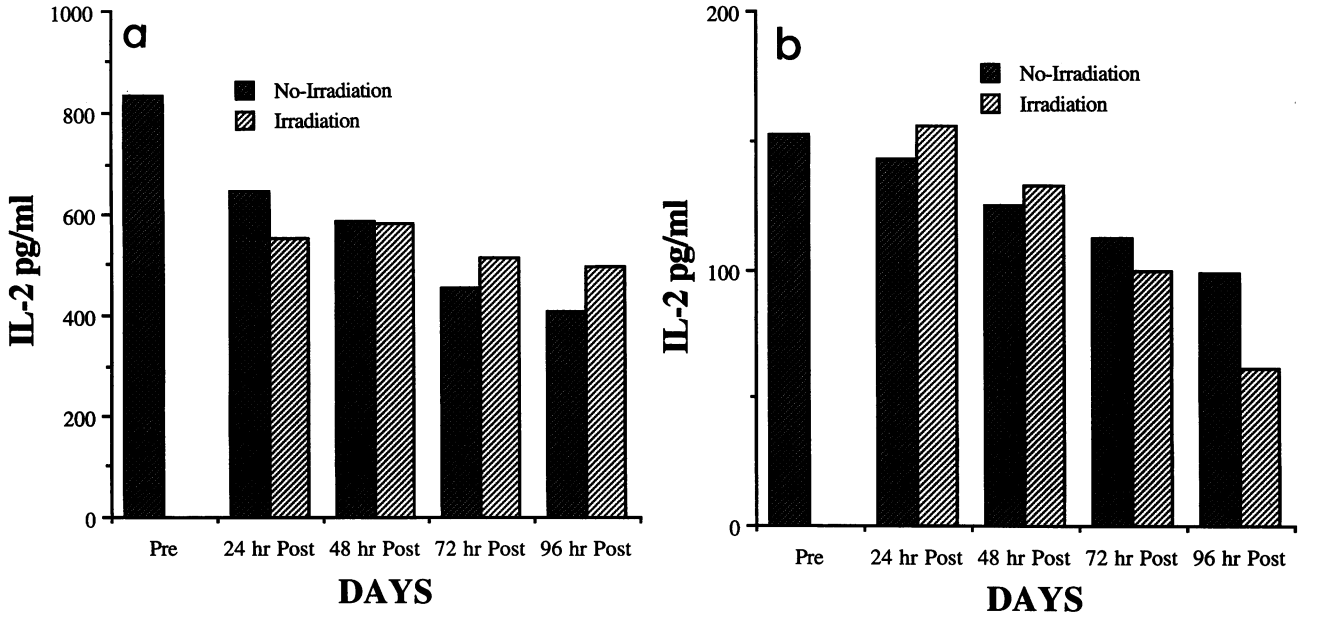

FIG. 5. Lethal irradiation posttransfection does not inhibit transgene expression. To determine the effect of irradiation on gene expression, the prostate cell line (a) and primary breast cells (b) were transfected and assessed for gene expression after lethal irradiation. Both cell types were transfected with optimal AAV plasmid-liposome complexes. On day 2 an aliquot of the cells was subjected to 6,000 rads with a ^{60}Co irradiator and kept in culture. Supernatants were collected 24, 48, 72, and 96 h after irradiation and tested for IL-2 levels. IL-2 levels are expressed as picograms per milliliter per 10^6 cells in 24 h of culture.

days in culture with RPMI 1640 medium and 10% FBS. Cells (5×10^6 to 10×10^6) were plated and transfected with 50 μg of AAV plasmid DNA and 100 to 200 nmol of liposomes (DDAB-DOPE or DDAB-cholesterol) to obtain a ratio of 1 μg of DNA to 2 nmol of liposome or 1 μg of DNA to 4 nmol of liposome. The cells were harvested 3, 7, or 10 days after transfection, the cell extracts were normalized by protein content, and CAT activity was measured by chromatographic assay.

As depicted, the lipid composition of the liposomes complexed to AAV plasmid DNA was varied, as was the ratio of DNA to liposome. The maximum level of expression was observed on days 2 and 3 in both total populations and subpopulations. Liposome DDAB-DOPE, either as 1 μg of DNA to 2 nmol of liposome or as 1 μg of DNA to 4 nmol of liposome, showed the most efficient transfection in all the populations. However, DDAB-cholesterol showed comparable efficiency with the CD3-selected population. A significant level of expression was detected up to day 14 (data not shown).

Southern blot analysis of AAV plasmid-liposome transfection. To analyze the molecular basis for the sustained expression observed in AAV plasmid-liposome transfection, Southern blot analysis of the chromosomal DNA from the Hirt extract was performed. DNA was isolated from both AAV plasmid- and standard plasmid-transfected R3327 cells at various time points. The chromosomal DNA was digested with *Bam*HI-*Hin*dIII to release the IL-2 cDNA insert (0.685 kb) and probed with a 0.685-kb IL-2 gene fragment. The data in Fig. 8 indicate that the IL-2 sequence was present in the AAV plasmid-transfected group up to day 25. Although there was some reduction in the density of IL-2 gene hybridization from days 9 through 25, the gene was clearly present even at 25 days posttransfection. In contrast, the 0.685-kb band of the IL-2 gene was detected on day 5 in cells transfected with the standard plasmid but there was no detectable IL-2 gene in the transfected cells at later time points.

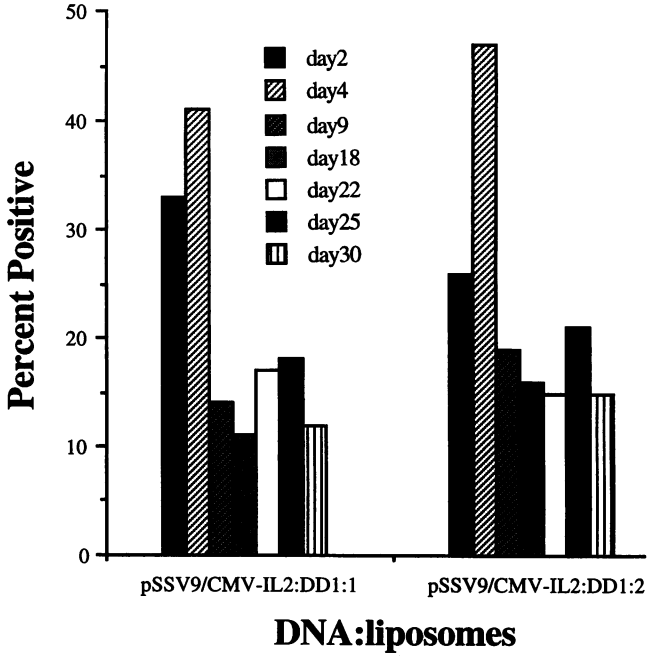

FIG. 6. Efficiency of transfection as measured by intracellular IL-2 gene expression. The prostate cell line was transfected with AAV IL-2 plasmid complexed with liposomes (DDAB-DOPE in a 1:1 or 1:2 composition). The DNA-liposome ratio was kept as 1 μg/3 nmol in both groups. Transfected cells were stained at various time points for intracellular IL-2 protein levels by a modified flow cytometry procedure as described in Materials and Methods. Approximately 10^6 cells were permeabilized and stained with anti-IL-2 monoclonal antibody followed by fluorescein isothiocyanate-conjugated anti-mouse $F(ab')_2$ antibodies. The cells were analyzed for positivity with a FACScan. The data are represented as percent positive cells expressing IL-2 protein. Untransfected cells were used as negative controls, and the values of controls were subtracted from the values of transfected groups.

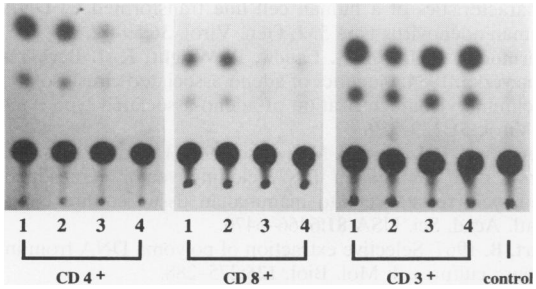

FIG. 7. AAV plasmid DNA complexed to liposomes transfects T lymphocytes. Primary T cells freshly isolated from peripheral blood were tested for transgene expression using AAV plasmid DNA-liposome complexes. T lymphocytes were fractionated as $CD3^+$, $CD4^+$, or $CD8^+$ populations with AIS MicroCELLector devices. The relevant cells were captured, and nonadherent cells were washed off. The adherent cells were removed from the devices after 2 days in culture with RPMI 1640 medium and 10% FBS. Cells (5×10^6 to 10×10^6) were plated and transfected with 50 μg of AAV plasmid DNA and 100 or 200 nmol of liposomes (DDAB-DOPE or DDAB-cholesterol) to obtain a ratio of 1 μg of DNA to 2 nmol of liposome or 1 μg of DNA to 4 nmol of liposome. The cells were harvested 3 days after transfection, the cell extractions were normalized by protein content, and CAT activity was measured by a chromatographic assay. Sample 1 was transfected with 100 nmol of DDAB-DOPE, sample 2 was transfected with 200 nmol of DDAB-DOPE, sample 3 was transfected with 100 nmol of DDAB-cholesterol, and sample 4 was transfected with 200 nmol of DDAB-cholesterol.

DISCUSSION

In these studies, the AAV plasmid which contained the transgene and AAV terminal repeats is used as a DNA vector and cationic liposomes are used as carrier molecules. It is demonstrated that the AAV plasmid DNA-liposome complexes efficiently transfected primary and cultured tumor cells. In addition, we demonstrate efficient transgene expression in freshly isolated T lymphocytes from human peripheral blood. AAV plasmid-liposome complexes induce high-level and long-term (up to 30-day) expression of the genes (Fig. 3), unlike the transient expression demonstrated by typical liposome-mediated transfection. Moreover, levels of expression observed with AAV plasmids are 10-fold higher than those with standard plasmid transfection, as shown in Fig. 2. Notably, sustained expression is demonstrated in the AAV plasmid-lipofected groups, as well as in the rAAV-transduced groups (Fig. 3).

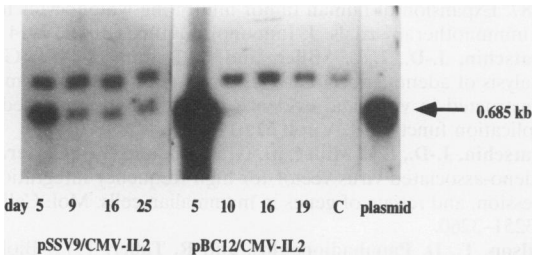

FIG. 8. Southern blot analysis of chromosomal DNA. Isolation of chromosomal DNA and Southern analysis were done as described in the text. The blots were probed with the 0.685-kb *Bam*HI-*Hin*dIII fragment of the IL-2 gene. Chromosomal DNA from both the AAV plasmid- and standard plasmid-transfected cells and untransfected control (C) was cut with *Bam*HI-*Hin*dIII. As a control, plasmids were cut with the same enzymes as the DNA from transfected cells. The migration position of 0.685 kb is indicated.

Most liposome-based gene delivery strategies, with the exception of pH-sensitive liposomes, have produced very low-level cellular expression of encapsulated genes. Recently, preformed liposomes containing synthetic cationic lipids have been shown to form very stable complexes with polyionic DNA. Furthermore, these cationic liposomes have been shown to mediate high-level expression of transgenes by delivering them to a wide variety of cultured cells (7, 15, 20). More recently, cationic liposome-DNA complexes have been used successfully to express transgenes in vivo after intravenous (30) or intraperitoneal (19) delivery. Unlike viral vectors, liposomes are noninfectious and appear nonimmunogenic in vivo. Furthermore, since preformed cationic liposomes readily complex with DNA, there may not be significant limitations to the size of the transgene that these liposomes can deliver. Despite all these advantages, liposomes have not been widely used because of low-level expression, particularly in primary cell types, and their inability to induce long-term expression. Long-term expression can be of advantage in gene expression in primary tumors for tumor vaccine studies as well as in gene modification of T cells or tumor-infiltrating lymphocytes. In this report, we overcome the duration of expression mediated by liposomes by inserting AAV terminal repeats in the plasmid construct used in transfection.

The combination of AAV plasmid and cationic liposomes not only transfected the cultured cell lines efficiently and yielded expression for over a period of 30 days but also transfected primary tumor cells and peripheral blood T cells. These data are noteworthy because most gene therapy strategies involve gene delivery to primary T lymphocytes or tumor cells. In addition, lethal irradiation did not affect the transgene expression either in cell lines or in primary tumor cells (Fig. 5). Gene-modified, irradiated tumor cells are widely used in tumor vaccine studies to induce an in vivo tumor immune response. These tumor vaccine studies require expression of transgenes after irradiation for at least 7 days posttransfection and irradiation, to induce a cytotoxic T-lymphocyte response in vivo. The data presented in this report demonstrate that AAV plasmid-liposome complexes may be of use in delivering transgenes into primary tumor cells for tumor vaccine therapies.

Gene therapy strategies have primarily relied upon transgene insertion into retroviral or DNA virus vectors. One of the disadvantages of the retroviral system is the inability to transfect nondividing primary cells (8). Although AAV vectors have features which make them preferable to retroviruses, including their ability to accommodate introns and the fact that they have never been associated with any human disease, they still require complicated processes for their production. They require the simultaneous presence of the wild-type AAV and adenovirus helper functions in order to proliferate (16, 17). The essential portion of rAAV for efficient transduction is the ITR; recombinant virus is made primarily to transport the AAV genome into the cell. When the plasmid containing the essential terminal repeats is combined with liposomes, the transfection process is simplified and high-level, long-term expression is achieved in both dividing and nondividing cell types, yet we avoid the complications involved with virus production. There was no difference in efficiency between optimal AAV transduction using current titers and maximal AAV plasmid-liposome transfection.

Cationic liposomes have been shown to mediate transient expression of standard plasmid DNA in mammalian cell types (7, 20). However, much lower efficiencies of integration into the host genome were observed in liposome-mediated transfection, which explains the short-term expression (22). Southern blot analysis of DNA from the AAV plasmid-liposome-

transfected group further substantiates the hypothesis that the long-term expression was indeed due to the presence of the transgene in these cells, which does not usually occur with standard plasmids lacking the ITR sequences (Fig. 8). Preliminary data from our laboratory with rAAV-infected cells showed a similar retention of the transgene as observed with AAV plasmid-liposome transfection (data not shown). The sustained presence of the transgene in AAV plasmid-transfected cells may result from integration as the molecular mechanism. The hypothesis that the AAV ITR plays a key role in the integration of the rAAV genome may also apply to the liposome-mediated transfection of the AAV ITR-containing plasmid. Studies to confirm integration as the molecular basis underlying these observations are under way. Investigations to determine the frequency and the site of integration in addition to detailed molecular analysis of liposome-mediated expression of AAV plasmid are also in progress.

In this report, it is demonstrated that viruses can be replaced by liposomes and efficient expression can be attained by utilizing a construct which includes viral elements responsible for both efficiency and duration. The AAV ITR may serve as an additional promoter to synergistically increase the efficiency of expression. In addition, the presence of the AAV ITR in the construct may also increase the integration frequencies of this plasmid into the host genome. Cationic liposomes are capable of transporting large amounts of plasmid DNA into the nucleus, which in combination with the plasmid consisting of the necessary elements for integration substantially increases the possibilities of integration. Furthermore, liposomes can deliver the plasmid DNA in the absence of any specific cell surface receptors and can replace the function of virus in gene delivery. In this manner production of virus for infection can be avoided, with the end results accomplished by a simple transfection process combining AAV plasmid and cationic liposomes. Additionally, plasmid DNA-liposome complexes can be delivered in vivo without any measurable toxicity (19, 24, 25, 30). In accordance with the observation, DNA concentration can be optimized to obtain maximum expression. The achievement of nonviral gene transfer into a wide variety of cell types ex vivo and in vivo would offer enhanced margins of safety and efficiency to any gene therapy protocol.

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