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Cancer Gene Therapy Using Plasmid DNA: Pharmacokinetic Study of DNA Following Injection in Mice

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ABSTRACT

The fate of plasmid DNA complexed with cationic lipids delivered intravenously in mice was evaluated at selected timepoints up to 6 months postinjection. Blood half-life and tissue distribution of plasmid DNA and potential expression in tissues were examined. Southern blot analyses of blood indicated that intact plasmid DNA was rapidly degraded, with a half-life of less than 5 min for intact plasmid, and was no longer detectable at 1 hr postinjection. Southern analyses of tissue demonstrated that intact DNA was differentially retained in the lung, spleen, liver, heart, kidney, marrow, and muscle up to 24 hr postinjection. After 7 days, no intact plasmid DNA was detectable by Southern blot analysis; however, the plasmid was detectable by the polymerase chain reaction (PCR) in all tissues examined at 7 and 28 days postinjection. At 6 months postinjection, femtogram levels of plasmid were detected only in muscle. Immunohistochemical analyses did not detect encoded protein in the tissues harboring residual plasmid at 1 or 7 days postinjection.

OVERVIEW SUMMARY

The present study evaluates the pharmacokinetic half-life and tissue distribution of plasmid DNA following intravenous injection in mice. This study extends the time frame of previous *in vivo* analyses to 6 months following i.v. injection. Injected mice exhibit no expression of the encoded gene as assayed by immunofluorescence. This represents the first systematic *in vivo* pharmacokinetic study of intravenously injected DNA complexed with cationic lipids, and is relevant to many gene therapy protocols utilizing direct injection of plasmid DNA plus lipids. The results provide a preliminary basis for the safe initiation of cancer immunotherapy clinical trials in which plasmid DNA is directly injected into tumors.

INTRODUCTION

DIRECT INTRATUMORAL INJECTION OF PLASMID DNA encoding foreign surface antigens or cytokines is a promising new approach for the treatment of cancer (Nabel *et al.*, 1992b, 1993; Vile and Hart, 1993). While a majority of malignancies arise in immunocompetent hosts, tumors escape host defenses. One theory by which tumor cells may evade immune surveillance is by altering their ability to express the major histocompatibility complex (MHC) class I molecules on their cell surfaces, thereby rendering themselves less visible to the immune system. Freshly isolated cells from naturally occurring tumors frequently lack or have decreased expression of MHC class I antigens (Schmidt *et al.*, 1981; Isakov *et al.*, 1983; Lampson *et al.*, 1983; Funa *et al.*, 1986). Studies have shown that direct

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gene transfer of MHC class I molecules into tumors may facilitate recognition of tumors by the immune system (Nabel *et al.*, 1992b; Nabel *et al.*, 1993).

Although an *ex vivo* approach of transfecting cells from individual patients for gene therapy has been successful, there are some limitations to the technique. These include the need to culture cells *in vitro* from each patient to avoid allogeneic tissue rejection, the tendency of cells to undergo phenotypic alteration in culture, the possibility of outgrowth of aberrant transformed cells, and the requirement for expertise to prepare cultured cells prior to reintroduction. Additionally, many gene therapy protocols require transduction with an integrating retroviral vector prior to reintroduction of the cells *in vivo*, thereby raising safety concerns.

In vivo introduction of recombinant DNA into tumors utilizing plasmid DNA expression vectors simplifies the gene transfer procedure and minimizes delays in treatment. Although tumors are the ultimate target of this gene transfer technology, the design of the current studies addresses the effects of DNA circulated inadvertantly throughout the body, which may result following direct tumor injection. The intravenous (i.v.) route of plasmid DNA injection was investigated because it provides the greatest potential for detection of systemic expression and manifestation of systemic toxicity.

Studies in mice have demonstrated the overall safety of i.v. DNA–cationic lipid injection as an *in vivo* gene transfer methodology (Nabel *et al.*, 1992; Stewart *et al.*, 1992). A previous clinical protocol utilized direct DNA injection into tumors for cancer immunotherapy trials (Nabel *et al.*, 1992b). The introduction of the gene encoding the heavy chain of an MHC class I molecule, HLA-B7, into patients with end-stage IV melanoma appeared to be safe. It resulted in transgene expression localized to the site of injection and was associated with tumor regression in 1 of 5 patients (Nabel *et al.*, 1993). We have expanded upon this initial finding and have entered a phase I clinical trial with a modified expression vector, pVCL-1005, encoding both the HLA-B7 heavy chain gene and the β2-microglobulin (β_2m) light chain gene. This plasmid contains a sequence encoding an internal ribosome entry site (IRES) between the HLA-B7 and β_2m cDNAs to coexpress them from the same promoter (Parks *et al.*, 1986; Jang *et al.*, 1988, 1989; Ghattas *et al.*, 1991). This differs from the plasmid used in the first clinical trial (Nabel *et al.*, 1993) in that the addition of an IRES and the gene encoding β_2m improves the expression level of the complete MHC class I molecule, containing both heavy and light polypeptide chains. In this preclinical study, the tissue distribution and half-life of pVCL-1005 plasmid DNA was analyzed over an extended period of time following i.v. injection in mice as VCL-1005, plasmid complexed with cationic lipids.

We found that the injected plasmid DNA persisted for at least 6 months postinjection following i.v. administration. Moreover, by immunohistochemical analysis, there was no detectable protein expression in those tissues that retained the greatest amount of plasmid. These results provided a basis for the expansion of human clinical trials using direct plasmid DNA injection.

MATERIALS AND METHODS

Plasmids

Supercoiled plasmid DNA was prepared by the method of Horn *et al.* (1995). Plasmid pCMVintLux (Manthorpe *et al.*, 1993) contains the human cytomegalovirus immediate early gene promoter (Thomsen *et al.*, 1984; Boshart *et al.*, 1985; Ghazal *et al.*, 1987) and intron A (Chapman *et al.*, 1991) at the 5′ end of a cDNA encoding the gene for firefly luciferase (De Wet *et al.*, 1987). Additionally, pCMVintLux possesses the SV40 small T intron and polyadenylation processing signal at the 3′ end of the cDNA for luciferase (Fig. 1A).

Plasmid pVCL-1005 contains the Rous sarcoma virus long terminal repeat (RSV-LTR) (Gorman *et al.*, 1983) promoter/enhancer that drives transcription of both the MHC class I human leukocyte antigen B7 gene (HLA-B7) and the chimpanzee β_2m gene (Fig. 1B). The HLA-B7 gene was originally derived from the plasmid pLJ-HLAB7, a gift from Dr. Alan Korman (Institut Pasteur, Paris). The cDNA for HLA-B7 heavy chain was originally cloned from a human B-cell library. The cDNA encoding chimpanzee β_2m was included to allow synthesis and expression of the complete major histocompatibility complex on the cell surface (Zamoyska and Parnes, 1988; Williams *et al.*, 1989). The clone encoding β_2m was a gift from Dr. Alejandro Madrigal, Stanford University. An internal ribosome entry site (IRES) sequence was placed between the HLA-B7 and β_2m cDNAs. The IRES was used to enable coexpression of the two genes from a single promoter in eukaryotic cells (Elroy-Stein *et al.*, 1989; Morgan *et al.*, 1992). To facilitate purification of the plasmid, and to obviate the use of ampicillin selection during the growth of the bacteria, the gene encoding ampicillin resistance (β-lactamase) was replaced with the gene encoding kanamycin resistance (aminoglycoside phosphotransferase) originally derived from the bacterial transposon *Tn*903 (Nomura *et al.*, 1978). pVCL-1005 also possesses transcript polyadenylation and termination signals from the bovine growth hormone polyadenylation sequence (Gordon *et al.*, 1983).

Plasmid pVCL-1004 contains the identical pBR322 backbone, kanamycin resistance gene, RSV-LTR promoter, and HLA-B7 sequences as pVCL-1005. However, it lacks the IRES and β_2m sequences, and the eukaryotic transcript is terminated with the SV40 small t antigen gene polyadenylation and termination sequence. Plasmid pRSV-BL contains the RSV promoter in a pBR322 backbone and has been previously described (Manthorpe *et al.*, 1993). Plasmid pRSV-lac Z encodes the gene for β-galactosidase (β-Gal) under control of the RSV promoter in a pBR322 backbone and has been previously described (Norton and Coffin, 1985).

In vitro *transfection and immunofluorescent staining*

The plasmids described above were transfected into L293 human embryonal kidney cells (β_2m positive/HLA-B7 positive) and UM-449 human melanoma cells (β_2m negative/HLA-B7 negative) using cationic lipid-based delivery. Cationic lipid reagents contain a net positive charge, and can bind to negatively charged molecules of DNA. Such reagents have been shown to facilitate transfection of plasmid DNA into cells cultured *in vitro* (Felgner *et al.*, 1987; Wang and Huang, 1989) and were used successfully *in vivo* in the clinical protocol for a previous cancer immunotherapy trial (Nabel *et al.*, 1992b). The transfection reagent used in the above clinical protocol, pVCL-1004 complexed with DC-Cholesterol:DOPE at a cationic lipid-to-DNA molar ratio of 3.0, was used for reference in this *in vitro* experiment. The cationic lipid DMRIE (1,2-dimyristoyl-

oxypropyl-3-dimethyl ammonium bromide; Felgner *et al.*, 1994) and the neutral lipid DOPE (dioleoyl phosphatidylethanolamine; Felgner, 1990) were formulated into liposomes at an equimolar ratio (1:1). With either plasmid, 2×10^5 cells in six-well plates were transfected with 20 μg of DNA, complexed with the appropriate lipid, in 2 ml of OptiMEM (GIBCO/BRL, Gaithersburg, MD) at 37°C. Four hours later, an additional 2 ml of OptiMEM/20% fetal bovine serum (FBS) was added to the cultures, and the cells continued to incubate for a total of 48 hr. Cells were then rinsed with 2–3 ml of phosphate-buffered saline (PBS), harvested in 2 ml of PBS containing 2 m*M* EDTA, and divided between two 12 × 75 mm polystyrene culture tubes (about 0.5×10^6 cells per tube). Cells were gently pelleted by centrifugation at 300 × g for 7 min at 4°C. Supernatants were discarded and one half of each culture was incubated for 1 hr on ice with either 100 μl anti-HLA-B7 monoclonal antibody (culture supernatant from hybridoma BB7.1, ATCC #HB56) for specific staining, or 100 μl of PBS, 5% fetal bovine serum (FBS) for nonspecific staining. All incubations and washes were done with chilled reagents and buffers containing 0.1% NaN_3. Cells were washed with 4 ml of PBS and incubated in the dark with R-phycoerythrin-labeled sheep anti-mouse IgG, $F(ab')_2$ fragment for 0.5 hr on ice. Cells were washed with PBS, resuspended in 0.2–0.4 ml of PBS/1% formaldehyde, and stored in the dark at 4°C until analyzed by flow cytometry.

Flow cytometric analysis was performed with a FACScan/LYSIS II system (Becton-Dickinson), using a 488-nm argon laser for excitation and the FL2 channel for detection at 585 nm. For each sample, 10,000 cells were counted, in triplicate. Data acquired from the triplicate determinations were presented in combined single-parameter fluorescence histograms, comparing for each sample the negative control distribution (nonspecifically stained cells) and test distribution (specifically stained cells). Cells of interest were identified by light scatter; data analysis gates were placed during analysis. In addition, the frequency of positive cells in each culture was determined by threshold analysis, setting the cut-off value for false positivity at 3%. This method may underestimate the positive population when there is an overlap between positive and negative distributions, but allows a semiquantitative evaluation of pVCL-1005 relative to pVCL-1004. Flow cytometry analysis was performed by Cytometry Sorting Specialities (San Diego, CA).

Preparation of VCL-1005 for in vivo *studies*

VCL-1005 is defined as the formulation consisting of a plasmid DNA, pVCL-1005, complexed with the cationic lipid DMRIE/DOPE at a DNA/lipid mass ratio of 5:1. VCL-1005 was prepared for administration by diluting the plasmid DNA pVCL-1005 with lactated Ringer's solution to a concentration of 1.0 mg/ml. The lyophilized lipid DMRIE/DOPE was reconstituted in a single vial with lactated Ringer's solution to a concentration of 0.96 mg/ml DMRIE and 1.12 mg/ml DOPE and diluted 1:4 with lactated Ringer's to give a final concentration of 0.192 mg/ml of DMRIE and 0.224 mg/ml DOPE. An equal volume of plasmid DNA at 1.0 mg/ml was mixed by gentle vortexing with diluted DMRIE/DOPE to form VCL-1005. This complex was used immediately for i.v. injection into mice. CMVintLux was prepared for injections in the same manner as VCL-1005.

Intravenous administration of VCL-1005

Plasmid DNA–lipid complexes were prepared as described and administered to ICR mice (6 weeks old, Harlan Sprague Dawley, San Diego, CA) as a single i.v. dose of 100 μl (50 μg of plasmid DNA) *via* the tail vein. Three female mice per timepoint were used for blood time course studies, and two males and two females per timepoint for tissue time course studies.

DNA isolation and analysis

Mice were sacrificed at the indicated times postinjection and exsanguinated by cardiac puncture. Blood was immediately mixed with EDTA to a final concentration of 100 m*M* in microfuge tubes and frozen in liquid nitrogen. The following tissues were then taken for subsequent analysis: bone marrow (femur), brain, heart, kidney, liver, lung, large intestine, small intestine, ovary/testis, spleen, and quadriceps muscle. Each tissue was placed in a microfuge tube and immediately frozen in liquid nitrogen. Tissues and blood were stored at −80°C until DNA extraction.

DNA was isolated from blood or tissues by overnight incubation at 65°C with 0.5 ml/tube of 0.5 mg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN) in 50 m*M* Tris-HCl pH 8.0, 100 m*M* EDTA, 100 m*M* NaCl, 1% SDS. Each mixture was subsequently extracted two to four times with equal volumes of buffered phenol, and then extracted with chloroform one to two times. DNA was precipitated at room temperature with 2 volumes of ethanol, pelleted at 12,000 × *g* in a microfuge for 10 min, rinsed in 70% ethanol, and dried at room temperature in a laminar flow hood. The DNA pellets were resuspended in 100 μl water with 0.1 mg/ml RNase A (Sigma, St. Louis, MO) and incubated at 37°C for 30 min. To remove digested RNA, DNA was again precipitated at room temperature with 2 volumes of ethanol, pelleted in a microfuge for 10 min, rinsed in 70% ethanol, and dried at room temperature in a laminar flow hood. The DNA pellet was rehydrated in 50–100 μl of water. OD_{260} readings were taken to determine DNA concentration. A 1-μg sample was run on a 0.8% agarose gel to visually confirm concentration of samples.

Southern blot analysis

Sample DNA (5–10 μg/lane) was separated by electrophoresis on 1% agarose in 1× TAE buffer (Ausubel *et al.*, 1987) at 35 V overnight. DNA was transferred by standard Southern blot procedures to Genescreen hybridization transfer membrane (Dupont NEN, Boston, MA) using a Posiblot pressure control apparatus under conditions recommended by the manufacturer (Stratagene, La Jolla, CA). Following transfer and UV cross-linking of DNA to the filter, membranes were prehybridized for 15 min to 3 hr in 1% bovine serum albumin (BSA), 7% SDS, 0.5 *M* $NaHPO_4$ pH 7.2, 1 m*M* EDTA at 65°C, and were then hybridized to the probe at 65°C overnight. The probe to pCMV-intLux was a random-primed ^{32}P-labeled 1,289-bp *Xba* I–*Eco* RV fragment of plasmid pRSV-luciferase. The probe to the HLA-B7 portion of VCL-1005 was a random-primed ^{32}P-labeled 1,058-bp *Bgl* II digest fragment. Blots were washed in 2× SSC, 0.1% SDS at room temperature for 10 min, and then in 0.5% BSA, 1% SDS, 40 m*M* $NaHPO_4$ pH 7.2, 1 m*M* EDTA

at 65°C for 1 hr with multiple changes of buffer (Ausubel *et al.*, 1987).

PCR analysis

Two sets of primers specific to plasmid sequences were made to amplify either a junction between the 3′ end of the RSV promoter and 5′ end of HLA-B7, or within the kanamycin resistance gene. HLA-1s sense and HLA-3a antisense primers produced an amplicon 491 bp in size. Kan 1s sense and Kan 3a antisense primers amplified a 426-bp fragment.

HLA 1s sense primer	5′-GCAACATGCCTTACAAG-GAG-3′
HLA 3a antisense primer	5′-TAGATCTGTGTGTTCCG-GTCC-3′
Kan 1s sense primer	5′-GGCAAGATCCTGG-TATCGGT-3′
Kan 3a antisense primer	5′-CGTACTCCTGATGATG-CATGG-3′

Primers were end-labeled in a kinase reaction containing 500 pmoles of oligonucleotide, 250 μCi (12.5 μl) [γ-^{32}P]ATP (Dupont-NEN, >6,000 Ci/mmole), 1 × T4 kinase buffer (70 m*M* Tris-HCl, pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol), and 10 units of T4 kinase (New England Biolabs) in a 50-μl reaction volume. The reaction was carried out for 30 min at 37°C and stopped with 1 μl of 0.5 m*M* EDTA.

Hot-start PCR amplification using Ampliwax beads (Perkin-Elmer) was performed in a Perkin-Elmer 9600 thermocycler using the following program:

1 × 94°C, 3′; 58°C, 110″; 75°C, 45″
5 × 94°C, 1′; 58°C, 110″; 75°C, 45″
25 × 94°C, 1′; 58°C, 110″; 75°C, 1′
1 × 94°C, 1′; 58°C, 110″; 75°C, 6′

The final concentrations for all PCR components in a 100-μl volume were as follows: 200 μ*M* of each dNTP, 20 pmoles of each labeled primer, 1 μg of extracted genomic DNA, and 2 units of *Taq* Polymerase (Stratagene) in 1× *Taq* Polymerase buffer (10 m*M* Tris, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin).

PCR reactions were run on nondenaturing 5.6% polyacrylamide gels with 1× TBE running buffer. Gels were run at 80 V for 14 hr or at 120 V for 7 hr. Twenty percent of the PCR reaction was loaded per lane with 1× stop dye. Gels were dried and exposed to Fuji RX autoradiographic film. Exposure times ranged from 30 min to 3 hr.

Immunohistochemical analyses

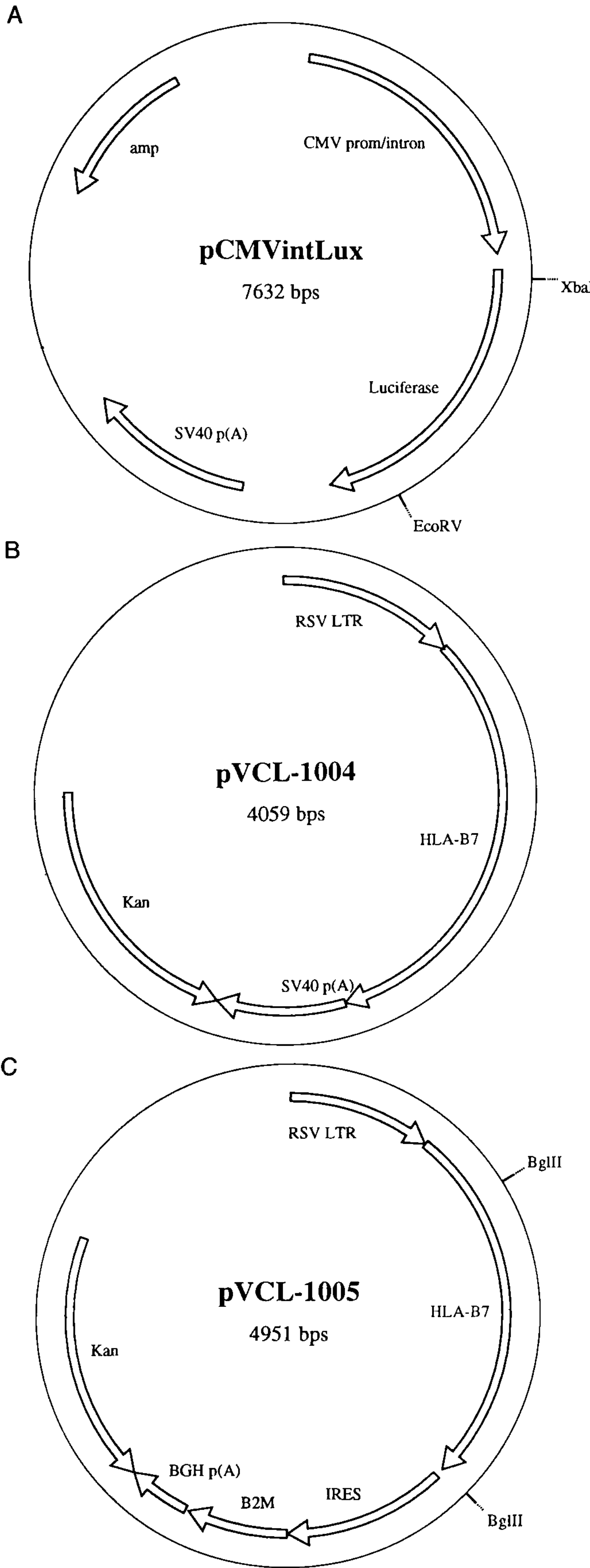
HLA-B7 expression was analyzed in ICR mouse liver, spleen, heart, lung, and kidney tissues using avidin-biotin immunoperoxidase staining at 24 hr ($n = 4$) and 7 days ($n = 4$) following i.v. injection of VCL-1005. Two animals injected with pRSV-BL served as negative controls. Anti-HLA-B7 monoclonal antibody (mAb) BB7.1 or H2K^d mAb SF1-1.1 (PharMingen, San Diego, CA), were used at a concentration of 7.14 μg/ml. Spleen, lymph nodes, and liver tissues from a HLA-B7/hβ_2m double-transgenic mouse were used as positive controls and were the generous gift of Dr. Per Peterson (The Scripps Research Institute, La Jolla, CA). Samples were embedded in O.C.T. (Tissue Tek, Elkhart, IN) and frozen in liquid nitrogen. Serial sections (6–8 μm) from two randomly selected areas of tissue were cut with a cryostat microtome, air-dried on glass slides at room temperature for 30 min, fixed in acetone for 15 sec at −20°C, and stored at −70°C. All subsequent steps were performed at room temperature. Liver tissue sections were treated with avidin/biotin blocking kit as described by the manufacturer (Zymed, South San Francisco, CA). Adjacent sections from each sample were incubated in 1% normal rabbit serum in PBS for 15 min to block nonspecific binding, and then incubated with biotinylated anti-HLA-B7 mAb or anti-H-2k^d mAb for 1 hr. After three 5-min washes in PBS, 4 μg/ml peroxidase-conjugated streptavidin (Jackson ImmunoResearch Labs, West Grove, PA) was applied for 30 min. After another wash, the peroxidase staining was developed using an AEC chromogen kit (3-amino, 9-ethyl carbazole, Biomeda, Foster City, CA) as recommended by the manufacturer. Sections were then counterstained with Mayer's hematoxylin (Zymed), washed in tap water, and mounted with Aqua-mount (Lerner Laboratories, Pittsburgh, PA).

RESULTS

Three plasmids were chosen for this study, pCMVintLux, pVCL-1004 and pVCL-1005 (Fig. 1). The first, pCMVintLux, contains the gene encoding firefly luciferase under the control of the CMV promoter. The second, pVCL-1004, contains the HLA-B7 heavy-chain cDNA under the control of the RSV 3′ LTR promoter and has been shown to express its gene product in transfected tumor cells *in vivo* (Nabel *et al.*, 1993). The third, pVCL-1005, is a modified version of pVCL-1004 designed to enhance expression of the MHC class I antigen HLA-B7. It contains two cDNAs transcribed as a single mRNA: HLA-B7 heavy chain plus chimpanzee β_2m light chain, under the control of a single RSV promoter (see Materials and Methods). The peptide sequences of chimpanzee and human β_2m are identical (Suggs *et al.*, 1981). The β_2m light chain is necessary for the complete MHC class I molecule to be expressed on the cell surface (Williams *et al.*, 1989). HLA-B7 is a MHC class I molecule which, if seen as allogeneic, may stimulate tissue rejection by identifying expressing cells as foreign to a nonexpressing host. Plasmid maps of pCMVintLux, pVCL-1004 and pVCL-1005 are shown in Fig. 1.

Expression in cultured cells

Prior to *in vivo* analysis, HLA-B7 cell-surface expression from pVCL-1004 and pVCL-1005 was initially assessed *in vitro* in transfected L293 embryonal kidney cells (β_2m$^+$) and UM-449 melanoma cells (β_2m$^-$). Expression was analyzed by flow cytometry using fluorescence-labeled secondary antibodies. The β_2m$^+$ L293 cells express endogenous HLA-B7 on the cell surface, as demonstrated by fluorescence on cells transfected with the negative control plasmid, pRSV-LacZ (Fig. 2A). However, transfection of L293 cells with plasmid pVCL-1004 (HLA-B7 only, Fig. 2B) or with pVCL-1005 (HLA-B7



and β_2m, Fig. 2C) enhanced HLA-B7 expression, as indicated by the population of cells that have greater fluorescence intensity. Transfection with pVCL-1005 appeared to generate about 1.5-fold more HLA-B7 positive cells than transfection with pVCL-1004.

To test the difference between bicistronic expression of HLA-B7 heavy chain plus β_2m light chain and monocistronic expression of HLA-B7, β_2m^- UM-449 melanoma cells were transfected with either pVCL-1005 or pVCL-1004. pRSV-LacZ transfection served as a negative control with which cells appeared to have little detectable expression of HLA-B7 (Fig. 2D). Transfection with pVCL-1004 generated only low levels of HLA-B7 expression (Fig. 2E). However, transfection with pVCL-1005, containing genes encoding both HLA-B7 and β_2m, elicited an increase in detectable levels of HLA-B7 cell-surface expression (Fig. 2F). The antibody to HLA-B7 is specific for the complete MHC class I molecule, including both light and heavy chains. Thus, the inclusion of the β_2m gene in the HLA-B7 expression vector resulted in increased HLA-B7 expression in cells which otherwise appear deficient in the expression of this protein.

Pharmacokinetics of plasmid in blood

Following the detection of efficient cell surface expression *in vitro* of HLA-B7 from the pVCL-1005 construct, experiments were carried out to determine the systemic distribution of plasmid DNA following i.v. tail injections in mice. An immune response, while desirable in the tumors to which the plasmid is targeted, could inadvertantly be elicited against normal tissues should the plasmid be expressed indiscriminantly throughout the body. Thus, a pharmacokinetic analysis of plasmid DNA in blood following i.v. injection was conducted using Southern blot analysis. Range-finding studies were initiated with the i.v. administration of 50 μg of pCMVintLux plasmid DNA, predominantly supercoiled, complexed with DMRIE/DOPE. Total DNA was isolated from blood at 1, 5, 15, 30, and 60 min postinjection and analyzed by Southern blot. The results indicated that supercoiled plasmid was not present, and that intact linear and relaxed circular plasmid forms were detected, but rapidly diminished between 1 and 30 min postinjection (Fig. 3A). Over the time course analyzed, the probe hybridized to material migrating faster than intact plasmid that gave the appearance of a smear on the blot. This indicated homologous but degraded plasmid sequences migrating at molecular weights

FIG. 1. A. Plasmid map of pCMVintLux. The cytomegalovirus immediate early gene promoter/enhancer and intron A control the cDNA encoding firefly luciferase (Lux). B. Plasmid map of pVCL-1004. The RSV-LTR promoter drives expression of the cDNA for HLA-B7. C. Plasmid map of pVCL-1005. The RSV-LTR promoter drives expression of 2 cDNAs, HLA-B7, and β_2m. The two genes are separated by an internal ribosomal entry site (IRES), which permits coexpression of the two genes from a single promoter in eukaryotic cells. SV40 int p(A), SV40 small t intron and polyadenylation signal; bGH p(A), bovine growth hormone transcription terminator and polyadenylation signal; amp, β-lactamase gene; kan, aminoglycoside phosphotransferase gene.

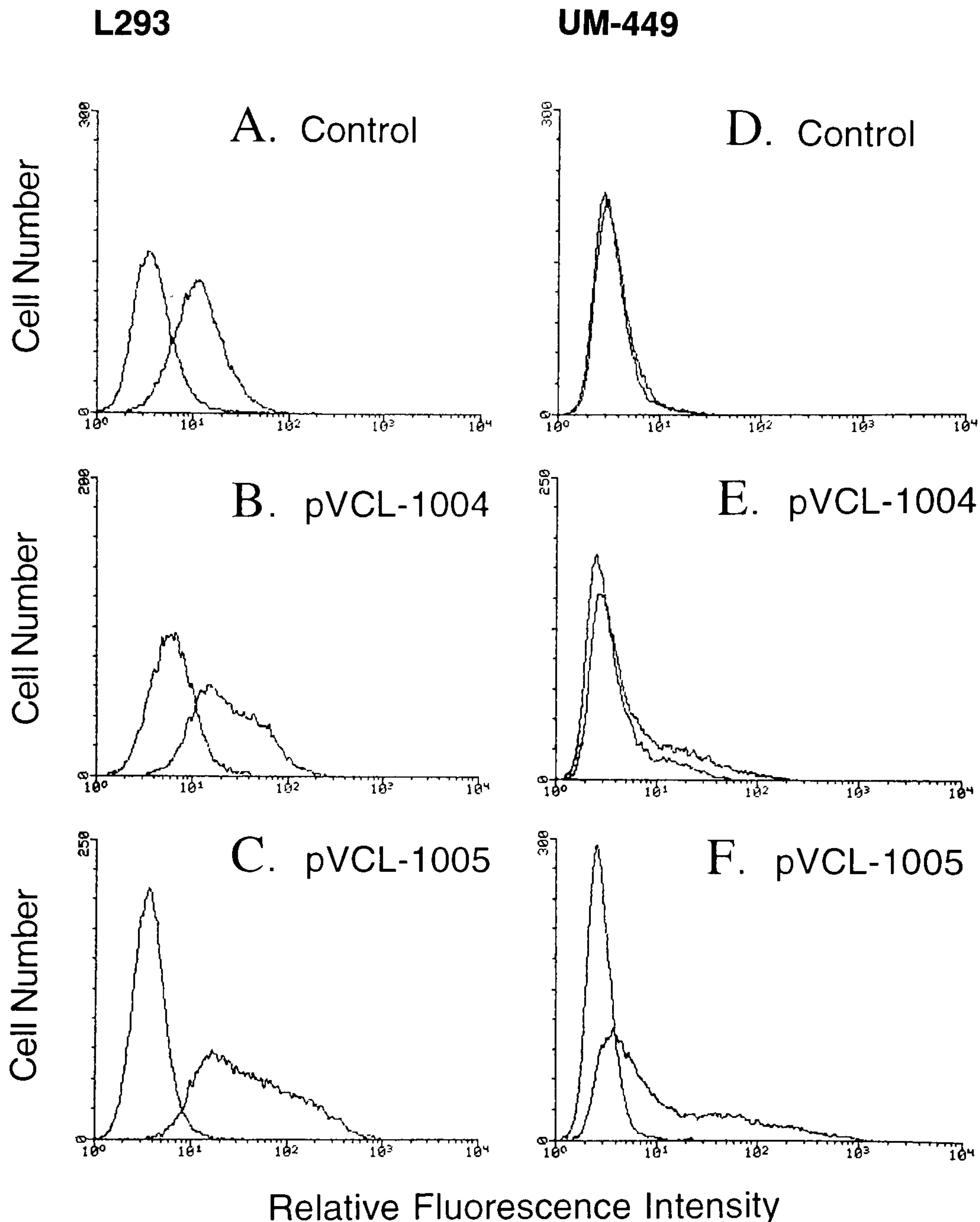


FIG. 2. *In vitro* HLA-B7 expression in transfected cells. L293 embryonal kidney cells (β_2m and HLA-B7 positive, A–C) or UM-449 melanoma cells (β_2m and HLA-B7 deficient, D–F) were transfected with the indicated plasmids and analyzed for HLA-B7 expression by flow cytometry following indirect fluorescent staining. Data acquired from the triplicate measurements of 10,000 cells are presented in combined single-parameter fluorescence histograms, comparing for each sample the negative control distribution (nonspecifically stained cells) and test distribution (specifically stained cells) as defined in Materials and Methods. Cell number is shown on the *y* axis, and relative fluorescence intensity is shown on the *x* axis. In each panel, the nonspecific (negative control) distribution is represented by the curves with the highest peaks, while specifically stained cells are represented by the curves shifted to the right or displaying deviation from the normal distribution. Greater shifts to the right indicate greater fluorescence intensities; more complete separation between the negative and the positive distributions is an indication of a higher frequency of positive cells. A and D. Data from cells transfected with pRSVLacZ DNA. B and E. Data from cells transfected with pVCL-1004, containing the gene for the HLA-B7 heavy chain only. C and F. Data from cells transfected with pVCL-1005, containing the genes for both HLA-B7 and the β_2m light chain.

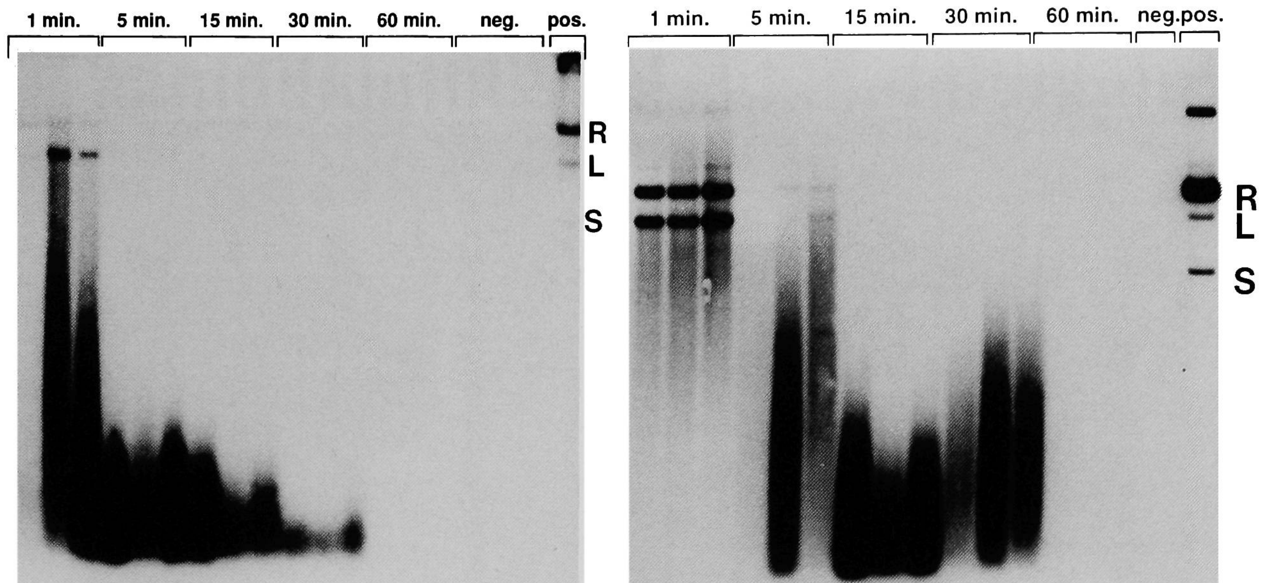


FIG. 3. Southern blot time course of plasmid in blood after intravenous injection. Following intravenous administration of 50 μg of plasmid DNA complexed with lipids, whole blood from three female ICR mice was isolated at each of the indicated times postinjection and DNA extracted. Five micrograms of each sample were separated on a 1% agarose gel. The transferred blot was probed with a random primed ^{32}P-labeled plasmid fragment. A. pCMVintLux injected. B. VCL-1005 injected. neg., negative control uninjected mouse blood DNA; pos., positive control uninjected mouse blood mixed with supercoiled plasmid DNA complexed with lipids, and then extracted; S, supercoiled plasmid; L, linear; R, relaxed.

lower than intact plasmid. The rapid appearance of this low-molecular-weight material showed that injected DNA was undergoing degradation within minutes. While degraded plasmid DNA sequences remained detectable at 30 min postinjection, the smear virtually disappeared from Southern blots by 60 min. As a positive control, supercoiled plasmid DNA was added to blood *in vitro*, immediately frozen, and extracted with the rest of the samples (Fig. 3A). This positive control showed that even brief exposure to blood resulted in the immediate conversion of the supercoiled plasmid to relaxed and linear forms. The results demonstrate that i.v. administered supercoiled plasmid DNA was immediately subjected to degradation, probably from nucleases present in the serum. Degradation or tissue entrapment led to the disappearance of most of the plasmid from blood within 1 hr, as assayed by Southern blot.

Consistent with the results of the above experiments using pCMVintLux, pVCL-1005 was found to be rapidly degraded following i.v. administration (Fig. 3B). The half-life of the intact (linear or relaxed circular) plasmid was less than 5 min, although degraded material was seen up to the 30-min timepoint. By 60 min, no HLA-B7 hybridizable DNA was detected by this method. The consistency between results indicated that the rapid disappearance of plasmid DNA from blood was independent of the sequence.

Pharmacokinetics of plasmid DNA in tissues

To determine the fate of plasmid taken up by tissues, a pharmacokinetic analysis of plasmid in tissue was conducted following i.v. administration of either CMVintLux (data not shown) or VCL-1005. Total DNA was isolated from bone marrow, brain, heart, kidney, liver, lung, large intestine, small intestine, ovary/testis, spleen, and muscle at timepoints ranging between 5 min to 6 months postinjection. At the earliest time point (5–10 min), intact linear and relaxed circular plasmid DNA was detected in all tissues by Southern analysis (data not shown). Because the animals were not perfused prior to isolation of tissues, this detectability was due, in part, to residual plasmid circulating in the blood. The highest level of residual plasmid was detected in the heart, kidney, liver, lung, and spleen, which are highly vascularized tissues. However, within 1 hr, plasmid DNA was detected mainly in the bone marrow, heart, kidney, liver, lung, spleen and muscle, but not detected in the brain, large intestine, small intestine and ovaries. Data from individual female mice is shown for VCL-1005 in Fig. 4A, with results that were identical to pCMVintLux. Results were also consistent with similar tissues in male mice. Plasmid DNA remained detectable by Southern blot for up to 24 hr postinjection, with the highest signal intensities found in the same tissues as at 1 hr postinjection (Fig. 4B). However, the exposure times for the autoradiograms were about 17 times longer with the 24-hr postinjection samples than with the 1-hr postinjection samples. Intact plasmid DNA remained detectable in tissues at 24 hr postinjection.

Southern analyses of 7-day postinjection samples of pVCL-1005 did not reveal any intact plasmid in tissues (Fig. 4C), as compared to the linearized positive control plasmid run in parallel. The nonspecific hybridization seen near the top of the blot was likely due to hybridization of mouse genomic DNA by the highly homologous human HLA-B7 probe, a consequence of long exposures of the autoradiogram. This hybridization was also seen in DNA from negative control mouse tissues (lactated Ringer's injected animal) in Fig. 4C, and therefore was not due to integration of plasmid into genomic DNA. The Southern blot technique has a sensitivity limit of about 1 pg of plasmid DNA, assuming the signal is in one band. Since 10 μg/lane of total

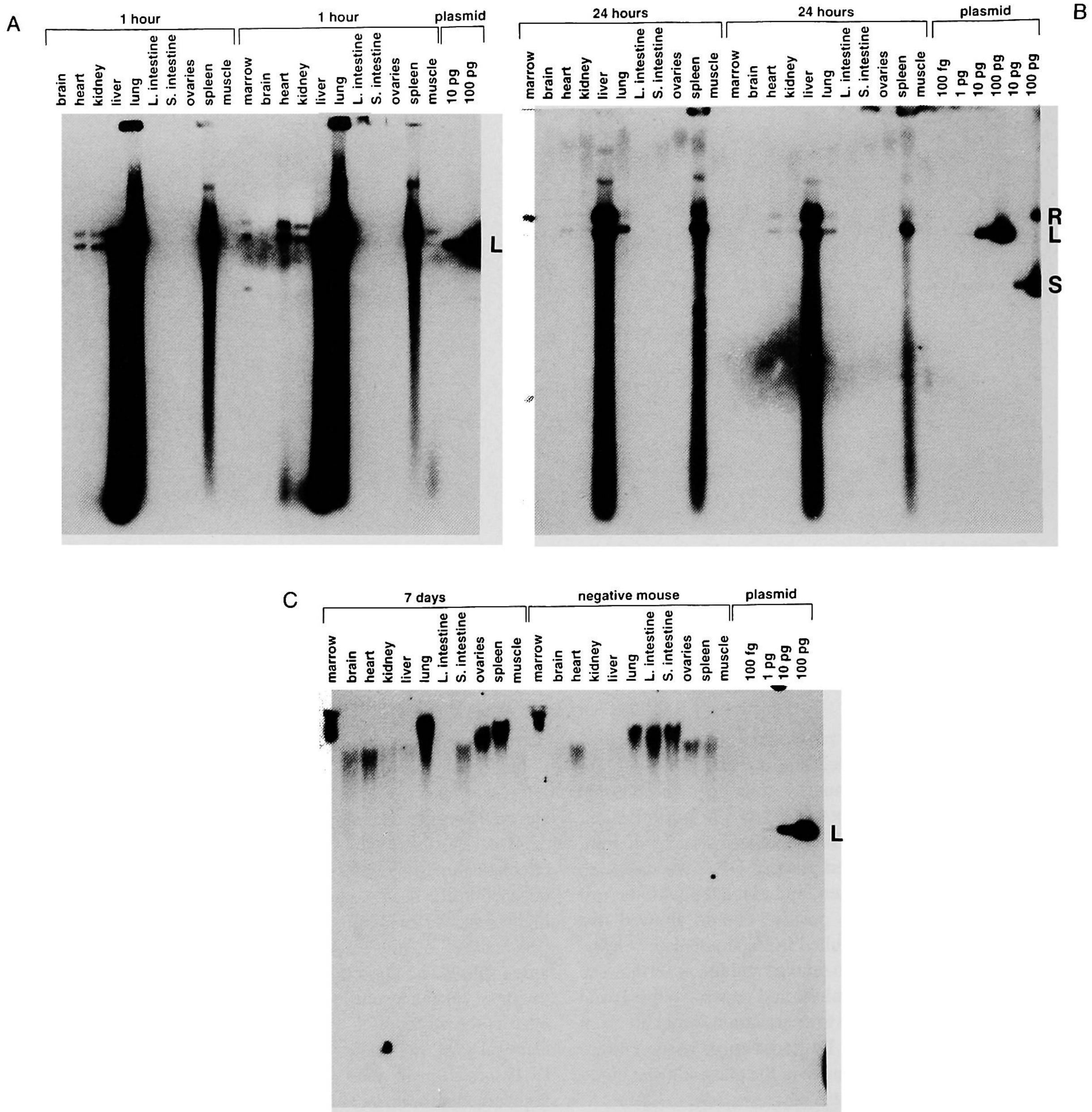


FIG. 4. Southern blots of tissue DNA following intravenous injection of VCL-1005. Following intravenous administration of 50 μg of plasmid DNA complexed with lipids, DNA from ICR female mice tissues was isolated at each of the indicated times postinjection. Results were consistent with similar tissues in male mice. Ten micrograms of each sample were separated on a 1% agarose gel. The transferred blots were probed with a random primed ^{32}P-labeled fragment of the HLA-B7 gene. Positive control was plasmid DNA mixed into negative mouse genomic DNA. S, Supercoiled plasmid; L, linear; R, relaxed. A. 1-hr postinjection. B. 24 hr postinjection. C. 7 days postinjection and uninjected.

genomic DNA was analyzed, detection of 1 pg would represent 0.15 copies of plasmid per genome.

PCR analyses

To detect levels of plasmid that were less than 1 pg, samples collected later than 24 hr postinjection were analyzed by PCR. PCR analysis was conducted using tissue samples collected 7 days, 28 days, and 6 months postinjection. ^{32}P-labeled, plasmid-specific primers were chosen to amplify either the junction between the RSV promoter and HLA-B7 heavy-chain gene, or within the kanamycin resistance gene. PCR was quantitated by comparing the band intensities of the samples to plasmid standards run in parallel in a 32-cycle reaction. Although Southern analyses indicated that no intact plasmid DNA was present in tissues tested at time points beyond 24 hr, PCR results show the presence of plasmid sequences in all tissues at 7 and 28 days postinjection (Fig. 5). At 7 days, the samples that amplified to the greatest extent were the marrow, heart, kidney, liver, lung, spleen, and muscle. These same tissues were the ones in which

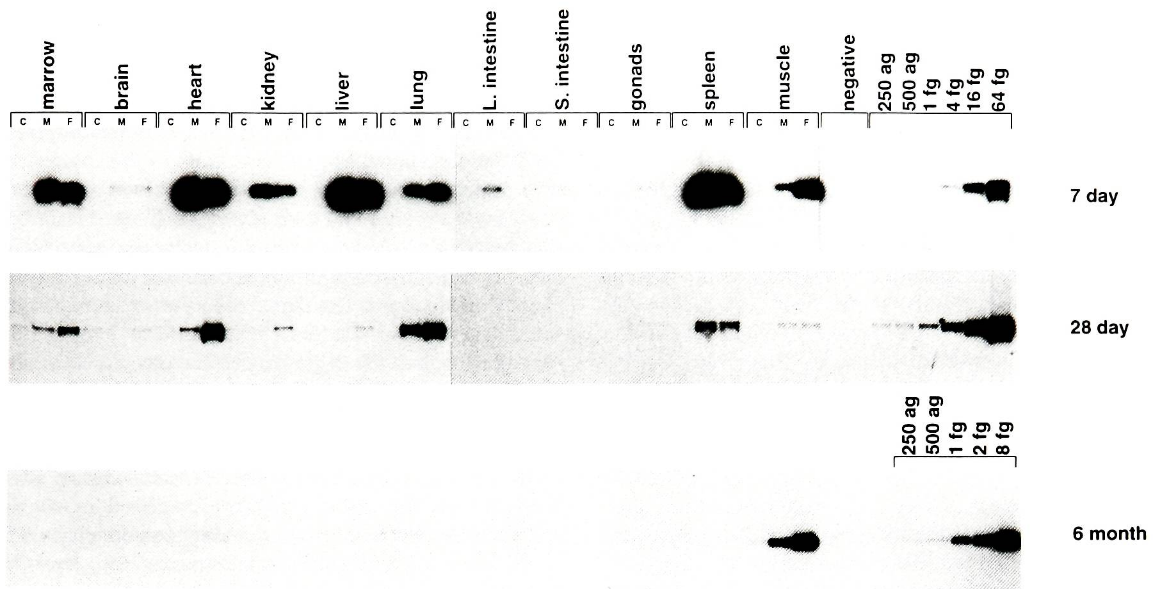


FIG. 5. PCR on extended timepoint postinjection tissue samples. Following intravenous injection of 50 μg of VCL-1005, 1 μg of genomic DNA from the indicated tissues was amplified by PCR containing ^{32}P-labeled primers specific for the plasmid. Samples were separated on a nondenaturing 5.6% polyacrylamide gel. Seven-day, 28-day, and 6-month postinjection results are shown. C. Negative control tissue DNA; M, male; F, female. Lanes designated as negative are PCR products from uninjected tissue DNA. Standards are PCR products from purified plasmid amplified in 1 μg of negative genomic DNA.

A C

B D

FIG. 6. Anti-HLA-B7 immunostaining of mouse tissues. Positive-control HLA-B7 heavy-chain/hβ_2m double transgenic mouse spleen (A) or liver (C) tissue were stained for HLA-B7 using the BB7.1 monoclonal antibody. Note extensive pericellular orange-yellow peroxidase staining. Spleen (B) and liver (D) tissues from ICR mice collected 24 hr following i.v. injection of VCL-1005 and stained with BB7.1 mAb were negative for HLA-B7 expression.

Southern blots detected the greatest amount of plasmid at earlier timepoints. The range of residual plasmid, as estimated from band intensities, was 1 fg/μg sample in the brain, intestines, and gonads, to about 64 fg/μg sample in the marrow, heart, liver, spleen, and muscle, representing approximately 250–16,000 copies/μg genomic DNA.

By 28 days, marrow, heart, kidney, liver, lung, spleen, and muscle still had the greatest amounts of amplifiable plasmid, although positive bands could be detected in all samples upon long autoradiographic exposure. The largest decrease from the 7-day samples appeared to be in the liver, and the smallest decrease in the lung. All 28-day tissues, with the exception of the lung, had less intense bands than the 7-day samples. The range of residual plasmid had diminished to less than 500 ag/μg in the brain, liver, intestines, and gonads, and to about 16 fg/μg genomic DNA in the marrow, heart, lung, and spleen. These amounts represent as much as a 128-fold decrease from the 7-day level, equivalent to about 125–4,000 copies/μg of genomic DNA. In a mouse haploid genome of 3×10^9 bp, this represents about 0.0015–0.096 copies/genome at 7 days, and about 0.0008–0.024 copies/genome at 28 days. Variability existed within the equivalent tissues among different animals, but the level of plasmid was at or below the level of 100 fg/μg genomic DNA in all samples by 28 days. At 6 months postinjection, pVCL-1005 plasmid was detected predominantly in muscle, at approximately 2–8 fg/μg genomic DNA, or about 0.012 copies/genome (Fig. 5). Because of background and variability, we could not discern if tissues had fewer than 0.0004 copies/genome.

Immunohistochemistry

To determine if the plasmid remaining in the tissues was being expressed, immunohistochemical analyses were carried out on the tissues that retained the greatest amount of plasmid after i.v. administration of VCL-1005, specifically the heart, kidney, liver, lung, and spleen. The specificity of the HLA-B7 antibodies used in the present study was determined by immunostaining sections from tissues of HLA-B7 heavy-chain/hβ_2m double transgenic mice. Spleens from these double transgenic animals exhibited very strong pericellular HLA-B7 staining (Fig. 6A) and livers were moderately stained (Fig. 6C). In contrast, no HLA-B7 immunostaining was seen in any of the heart, kidney, lung (data not shown), spleen, and liver (Fig. 6B,D) tissues collected from mice 1 and 7 days after injection with VCL-1005. Therefore, despite the fact that the heart, kidney, liver, lung, and spleen contained some of the highest levels of intact pVCL-1005 plasmid DNA by Southern and PCR analyses at 1 and 7 days, none of these tissues was positive for HLA-B7 expression.

DISCUSSION

The consequence of direct intratumoral plasmid DNA injection may be the inadvertant introduction of DNA into other tissues *via* the bloodstream. Using plasmids encoding either a reporter gene or a complete MHC class I molecule, it was demonstrated that most of the plasmid DNA administered i.v. was rapidly degraded and cleared from blood within minutes and from tissues within hours of administration. The most significant decrease in detectable plasmid occurred during the first hour following injection.

Although the predominant plasmid conformation present in the DNA–lipid complex prior to injection was supercoiled, the only detectable intact forms in either the blood or tissues after injection were linear and relaxed circular DNA. Plasmid DNA was apparently subjected to serum nucleases in the blood almost immediately upon i.v. injection. The half-life of intact plasmid in blood was less than 5 min. By Southern blot analyses, intact plasmid was present in blood as long as 15 min postinjection, but only degraded plasmid was present at 30 min. By 60 min, even the degraded material had been cleared from the blood, and there was no detectable retention of intact plasmid DNA by Southern blot analysis (sensitivity limit = 1 pg).

Specific tissues retained plasmid DNA immediately after i.v. injection, but the amount greatly diminished in all tissues within hours. One hour after i.v. administration, plasmid DNA was detected predominantly in the lung, spleen, liver, heart, kidney, marrow, and muscle. By Southern analysis, there was no detectable plasmid in the brain, large intestine, small intestine, or gonads at the 1-hr timepoint. Southern analysis also demonstrated that plasmid DNA remained in the liver, spleen, lung, marrow, and muscle, although at diminished levels, up to 24 hr postinjection. At the 7- and 28-day timepoints, which could only be analyzed with the increased sensitivity of PCR, there was detectable plasmid in all tissues examined. However, most of the residual plasmid was detected in the same tissues as had been previously determined by Southern blot analysis. PCR analysis at the 6-month timepoint revealed that only muscle had any significant levels of plasmid above background. These results demonstrated that the amount of plasmid DNA remaining in the tissues following i.v. injection of 50 μg plasmid drops significantly with less than 0.15 copies/genome within 24 hr, less than 0.096 copies/genome at 7 days, less than 0.024 copies/genome at 28 days, and less than 0.012 copies/genome at 6 months.

Occasionally, plasmid DNA was detected in tissues other than the marrow, heart, kidney, liver, lung, spleen, and muscle in animals beyond the 28-day timepoint (data not shown). These findings were variable among animals and among different experiments, and probably represent contamination. Longer autoradiographic exposures at each timepoint revealed that all tissues amplified at very low levels of approximately 0.0003–0.0004 copies/genome. These results were frequently indistinguishable from negative controls and thus were considered background. This low level may also represent nonspecific amplification from mouse genomic DNA, or variable low-level retention of plasmid in mouse tissues.

Although the amount of plasmid in the 6-month muscle samples appeared to be greater than in the 28-day samples, this does not represent an accumulation of plasmid in the muscle. More likely explanations are that variability exists among animals from different experiments, and that the PCR quantitation is only an estimate. Because skeletal muscle is one of the tissues that can be transfected *in vivo* upon direct intramuscular (i.m.) injection (Wolff *et al.*, 1990, 1991; Acsadi *et al.*, 1991; Jiao *et al.*, 1992; Davis *et al.*, 1993a,b), and plasmid DNA can be detected for greater than 1 year following direct i.m. injection,

the continued presence of intact plasmid in muscle might be expected following i.v. injection.

While it was clear that some plasmid DNA was detectable by PCR for at least 6 months postinjection, there was no detectable HLA-B7 protein expression at earlier timepoints in tissues where the plasmid was observed to accumulate and when maximal expression was expected. Since immunostaining analysis is relatively insensitive compared to PCR, further studies on mRNA transcript levels following i.v. plasmid injection will need to be addressed. The observation that HLA-B7 protein expression levels were too low to be detected by immunostaining may suggest that levels were likely to be too low to elicit an immune response when administered systemically. Additionally, luciferase assays of mouse tissues following i.v. pCMV-intLux injection did not result in luciferase expression in any tissues (unpublished observations).

In the present study, we have used a bicistronic plasmid, pVCL-1005, in which the cDNAs for HLA-B7 and β_2m are under control of a single RSV promoter. The utility of this gene combination is demonstrated by higher MHC class I expression levels obtained when using this plasmid to transfect cells *in vitro* compared to using a plasmid encoding the HLA-B7 heavy chain alone. The plasmid pVCL-1005, which encodes both HLA-B7 heavy chain and β_2m light chain, appeared to increase HLA-B7 surface expression in cells which contain low levels of endogenous β_2m. Additionally, even in cells that express endogenous β_2m, such as L293 cells, HLA-B7 expression levels increased following transfection with pVCL-1005.

Current injection technology does not permit exclusive transfection of tumor tissue. However, histological analyses of the liver, spleen, heart, and lung at timepoints at which peak expression was expected, did not reveal expression of HLA-B7 protein. It is possible that the expression levels were below the limit of detection with this technique. *In vivo* expression of this plasmid has been shown indirectly in BALB/c mice, which gave rise to antibodies to both heavy and light chains of the HLA-B7 protein following direct i.m. injection pVCL-1005 (unpublished observations). However, in a companion safety study, none of the animals that received an i.v. administration of VCL-1005 exhibited signs of autoimmune attack (see accompanying paper by Parker *et al.*).

The intravenous injection of DNA with cationic lipids has been reported previously to result in expression of a chloramphenicol acetyltransferase (CAT) reporter gene product in many mouse tissues 48 hr postinjection (Zhu *et al.*, 1993). Moreover, these investigators were able to detect plasmid DNA sequences by PCR 63 days postinjection in lung, heart, and spleen. Although the PCR results from the present study were consistent with DNA detection results in terms of plasmid localization, expression of plasmid encoded HLA-B7 was not found in tissues following i.v. administration of VCL-1005. Future investigations should elucidate the contributions made by factors such as mRNA and protein stability to the levels of protein expressed *in vivo* (Ledley and Ledley, 1994).

This study represents the first systematic *in vivo* pharmacokinetic study of intravenously injected DNA complexed with cationic lipids and was presented in support of ongoing clinical trials. It is relevant to many current and future gene therapy trials utilizing direct injection of plasmid DNA and should facilitate understanding of the safety of this procedure.

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