Poly(D,L-Lactic-co-Glycolic Acid) microsphere delivery of adenovirus for vaccination

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Dedicated to Prof. Antoine (Tony) A. Noujaim on the occasion of his 70th birthday, in recognition of his outstanding contributions to radiopharmacy, diagnostic oncology and the immunotherapy of cancer.

ABSTRACT - Purpose: To study the effect of encapsulation of recombinant adenovirus type 5 encoding β-galactosidase (Ad5-βgal) in poly (D,L-lactic-co-glycolic acid) (PLGA) microspheres on viral delivery to professional antigen presenting cells (APCs) *in vitro*, viral dissemination *in vivo*, and induction of protective immune responses *in vivo*. **Methods:** PLGA microspheres containing Ad5-βgal were prepared by a double emulsion solvent evaporation method. Encapsulation efficiency, *in vitro* release profile, *in vitro* cellular uptake and *in vivo* biodistribution of Ad5-βgal loaded PLGA microspheres were determined using ^{125}I-labeled Ad5-βgal (^{125}I-Ad5-βgal). To evaluate the potential of PLGA microsphere delivery of Ad-βgal for induction of antigen-specific immune responses *in vivo*, Balb/c mice were immunized with the subcutaneous injection of the formulations then splenocytes of the immunized mice were assayed for cytotoxic T lymphocyte (CTL) activity against a variety of target cells in a ^{51}Cr-release assay. Anti-βgal antibody responses were assessed in the sera of the immunized mice by enzyme linked immunosorbent assay (ELISA). The effect of encapsulated Ad5-βgal immunization on protection against a tumor challenge was tested in a murine artificial metastatic lung tumor model with βgal-expressing tumor cells, CT26.CL25. **Results:** PLGA microspheres encapsulated Ad5-βgal with 24.8 ± 1.4 % encapsulation efficiency and 11.4 ± 3.6 % of the encapsulated virus retained the functional activity. *In vitro* release study showed slow release (15% in 11 days) of the virus from the microspheres. PLGA microsphere delivery of Ad5-βgal resulted in enhanced uptake of the virus by APCs with an increase in the transgene expression *in vitro*. Administration of the virus in the encapsulated form resulted in substantially decreased viral dissemination to remote organs and tissues as compared to the free virus. Encapsulated virus were capable of eliciting antigen-specific CTL as well as antibody responses against βgal and induced protective immune responses against lethal tumor challenge at a significantly lower infectious viral dose as compared to the free virus. **Conclusion:** PLGA microsphere with Ad5-βgal enhances the delivery of virus to APCs with reduced viral dissemination in other organs and induces protective antigen-specific immune responses against viral encoded transgene.

INTRODUCTION

Recombinant adenoviruses, originally used as vehicles for gene replacement therapy (1, 2), have attracted a great deal of interest as vaccine carriers for the delivery of genes derived from pathogen (3-5) or tumors (6) for a number of reasons. The adenoviral genome is well characterized and quite easy to manipulate. Adenoviruses can be grown to high titer in available cell lines, and methods for purification and production are well established, which facilitates their development for clinical use. Most of the past efforts focused on human adenoviruses, such as those of the common serotype 5 (Ad5), which cause only mild upper respiratory tract symptoms upon natural infections. By deletion of vital regions of the viral genome the adenoviral vectors can be rendered replication-defective, which enhances their predictability and reduces undesired side effects (4). The main characteristic of adenoviruses as vaccine carriers is their ability for transfection of professional antigen presenting cells (APCs) such as dendritic cells (DCs). Replication-incompetent adenoviral vectors have been shown to efficiently transduce professional APCs including DCs and induce strong and sustained transgene product-specific immune responses upon delivery of a single dose given systemically (7-9). However, the clinical applications of adenoviral vectors have been limited due to problems of vector-mediated

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immunogenicity, lack of specific tissue-specific targeting of the vectors and induction of the innate toxicity following intravascular application. Intravascular administration of Ad5 can induce innate toxicity which is characterized by complement activation, cytokine release and subsequent vascular damage leading to a systemic inflammatory response (10, 11). Anti-vector immunity is another challenge associated with the use of adenoviral vectors. Indeed the effectiveness of vaccine can be reduced by undesirable immune responses against the surface capsid proteins of the vectors which can be preexisting or induced following the first inoculation with adenoviral vector (12, 13). Finally due to their broad host cell specificity, adenoviruses administered *in vivo* will infect a variety of cells including epithelial cells, liver cells and lung cells. This can lead to immune attack of the infected cells leading to inflammation in various organs (14). Selective delivery of adenoviruses to professional APCs may overcome problems associated with viral dissemination and undesired immune responses.

Professional APCs such as DCs play a crucial role in induction of T cell responses against antigens (15). Various studies in animal models have shown that vaccination with DCs tranfected *ex vivo* with genes encoding antigens (16, 17), or pulsed *ex vivo* with peptides, proteins, or tumor-derived RNA (18-20) prime cytotoxic T lymphocyte (CTL) responses *in vivo*. Delivery of the gene encoding the antigen of interest to APCs genetically modifies these cells and transgene expression provides a constant supply of antigen. Direct introduction of genes encoding antigens into professional APCs, in particular DCs, has been proposed to be an ideal strategy for induction of strong T cell immune responses (17). Delivery systems suitable for targeted delivery of antigens or the gene encoding the antigen of interest to professional APCs *in vivo* are advantageous, since they eliminate the extensive efforts required for *ex vivo* generation of antigen-primed autologous APCs for each patient. Poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres are promising delivery systems for passive targeting to professional APCs since they are rapidly phagocytosed by APCs (21). These polymeric microspheres have been used to deliver peptides, proteins (22-26) and plasmid DNA (27-29) to APCs by a variety of immunization routes. PLGA microspheres are also appealing as a vaccine delivery vehicles since they are biodegradable and suitable for use in humans (30).

In this study we have investigated the effect of encapsulation of Ad5 encoding β-galactosidase (Ad5-βgal) in PLGA microspheres on viral delivery to professional APCs *in vitro*, viral dissemination *in vivo*, and induction of protective immune responses *in vivo*.

MATERIALS AND METHODS

Animals and Cell lines

Female Balb/c (H-2^d) and C57BL/6 (H-2^b) mice aged 6 to 8 week were purchased from Jackson Laboratory (Bar Harbor, ME). CT26.WT (H-2^d), an undifferentiated colon carcinoma from Balb/c mice and CT26.CL25, a β-gal-transfected CT26.WT (31) were kindly provided by Dr. N.P. Restifo (NIH, Bethesda, MD). CT26.WT and EL4 (a mouse T-lymphoma: H-2^b) cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY). CT26.CL25 was grown in the same medium containing 400 μg/ml G418 (GIBCO BRL, Gaithersburg, MD). An Ad E1-transformed human embryo kidney cell line 293(32), a mouse macrophage cell line J774A.1(33), and a mouse mammary carcinoma cell line 410.4 (34) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS.

Preparation of human dendritic cells (DCs)

Human dendritic cell cultures were established *in vitro* from peripheral blood of normal donors following established procedures (35, 36) using granulocyte monocyte stimulating factor (GM-CSF) and interleukin-4 (IL-4). DCs were shown to be MHC class II^+, $CD80^+$ and $CD86^+$, $CD14^-$ by FACS analysis using monoclonal antibodies specific for these markers.

Adenovirus

The recombinant adenovirus type 5 expressing *Escherichia coli* β-galactosidase (Ad5-βgal) was provided by Dr J. Elliott (University of Alberta, Edmonton, AB, Canada)(37). Viruses were amplified by growth in 293 cells and purified by two rounds of CsCl density gradient centrifugation (38), dialyzed against phosphate-buffered saline (PBS) supplemented with 10% glycerol and stored at – 70° C. The biological activity of each viral

preparation was quantified by a limiting dilution plaque assay using 293 cells as targets and is expressed as plaque forming units (PFU) (39).

Radioiodination of adenovirus was done by the iodogen method (40). About 10 μg of iodogen (tetrachloro-diphenylglycouril) was coated to the bottom of a test tube. A sample of adenovirus (2 X 10^9 pfu) in PBS (50μl) was added to the test tube followed by 2000μCi of $Na^{125}I$ (Amersham, Oakville, Canada) in PBS (40μl). After a reaction time of about 45 minutes, radioiodinated Ad5-βgal was separated from the free radioiodine using gel filtration on a Sephadex PD-10 desalting column (Pharmacia, Uppsala, Sweden). The specific activity of labeled adenovirus ranged between 2.88 to 5.92 μCi /10^9 viral particles.

Peptides

Synthetic peptides ICPMYARV ($\beta gal_{497-504}$) and TPHPARIGL ($\beta gal_{876-884}$) representing the H-2 K^b and H-2 L^d restricted CTL epitopes of βgal respectively (41, 42) were synthesized by the solid phase method (43) using a automated peptide synthesizer and were provided by Dr. David Wishart (University of Alberta).

Encapsulation of recombinant adenovirus in PLGA microspheres

PLGA microspheres containing Ad5-βgal were prepared by a double emulsion solvent evaporation method (44, 45). Briefly, 3 X 10^9 PFU Ad5-βgal in 100 μl of PBS with 10% glycerol were emulsified in 500 μl of dichloromethane containing 100 mg of PLGA (lactic to glycolic acid ratio: 50:50; molecular weight: 6,000, Birmingham polymers, Birmingham, AL) by vortexing. The resulting primary emulsion was added to 2 ml of 9% (w/v) polyvinyl alcohol (PVA, molecular weight 31,000-50,000, Aldrich Chem., Milwaukee, WI) and vortexed to form a double emulsion. This emulsion was then added dropwise into 8 ml of 9% (w/v) PVA and was continuously stirred for 3 h to evaporate dichloromethane. The microspheres were collected by centrifugation at 9000 X *g*, washed 3 times with PBS, and resuspended in PBS at a concentration of 50 mg/ml. Encapsulation efficiency of Ad5-βgal in PLGA microspheres was determined using ^{125}I-labeled Ad5-βgal (^{125}I-Ad5-βgal). Encapsulation efficiency was % of ^{125}I-Ad5-βgal encapsulated in PLGA microspheres vs. unencapsulated ones.

Particle size analysis

Particle size of the PLGA microspheres was routinely determined by dynamic light scattering (Brookhaven Instruments, Holtsville, NY). In addition, the surface morphology and particle size of selected samples were also determined by scanning electron microscopy (SEM). For this, PLGA microspheres (5.0 mg) were attached to a metal stub and placed in a sputter coater (Edwards, S150B, Sussex, UK) for 40 s to produce a gold coating of about 30 mm thickness. The coated samples were viewed under a Hitachi S-2500 scanning electron microscope (Hitachi, Tokyo, Japan) at a magnification of 10,000.

Release of encapsulated Ad5-βgal from PLGA microspheres in vitro

PLGA microspheres (5 mg) containing ^{125}I-Ad5-βgal (0.2μCi) were suspended in PBS (1 ml) and shaken in a water bath at 37°C. At predetermined time intervals, the supernatant was collected by centrifugation (12,000 X *g* for 10 min). The amount of virus released from micropheres was determined by measuring the radioactivity (CPM) of supernatant and pellet (microspheres) respectively. The percentage of viral release was calculated as follows: $[CPM_{supernatant} / CPM_{pellet} + CPM_{supernatant}]$ X 100.

Cellular uptake of Ad5-βgal by J774A.1 cells

Murine macrophage cells (J774A.1) and mouse mammary carcinoma cells (410.4) were cultured in 24-well plates in 1ml of DMEM supplemented with 10% FBS for 24 h at a cell density of 5 x 10^5 cells/well. PLGA microspheres (250 μg) containing ^{125}I-Ad5-βgal (0.01 μCi; 1.75 X 10^6 PFU) or same amount of free ^{125}I-Ad5-βgal in 0.5 ml medium were added to each well. After 24 hours of co-culture, the free microspheres were completely removed by washing with PBS for 5 times. The cells were then lysed with 1% SDS. The cellular uptake of Ad5-βgal was determined by measuring the radioactivity (CPM) of the cell lysate.

βgal expression in DCs and J774A.1 cells

DCs or J774A.1 cells were co-cultured with free Ad5-βgal (2.5x10^6 PFU), PLGA microspheres (250 μg) containing Ad5-βgal (2.5x10^6 PFU), or empty

PLGA microspheres (250 μg). The transgene expression was monitored by histochemical staining for β-galactosidase activity at various time points. Briefly, the cells were fixed in 0.25% glutaraldehyde (v/v) for 15 min, followed by incubation in X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) staining solution (10 mM sodium phosphate pH 7.0, 1mM $MgCl_2$, 150 mM NaCl, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$ and 1 mg/ml X-gal) at 37°C for 1.5 to 2 hr. The cells were examined under light microscope and a positive blue staining indicated βgal expression.

Biodistribution of microsphere-encapsulated ^{125}I-Ad5-βgal in mice

C57BL/6 mice (n=3) were subcutaneously injected with ^{125}I-Ad5-βgal (1.2 μCi containing 2 x10^7 viral particle per mouse) either as free virus or encapsulated in PLGA microspheres (3.5 mg). Mice were sacrificed at pre-determined intervals. Blood and various organs including liver, lungs, spleen, kidney, thyroid and brain were collected. The radioactivity (CPM) in tissue samples was determined by γ-scintillation counting.

Immunization and CTL assay

Balb/c mice (n=4) were immunized twice 14 days apart by subcutaneous injection of PLGA microspheres (10 mg) containing Ad5-βgal (1 X 10^8 PFU), free Ad5-βgal (1 X 10^8 PFU), or empty PLGA microspheres (10 mg) suspended in PBS (200 μl). Spleens were removed 14 d after second vaccination. Splenocytes (5 X 10^6 cells) from the immunized mice were co-cultured in 24-well plates with irradiated (3,000 rad) syngeneic splenocytes (2.5 X 10^6), previously pulsed (3 hours) with $βgal_{876-884}$ peptide (50 μg/ ml). After 5 days of culture, the viable splenocytes were assayed for CTL activity against a variety of target cells in a ^{51}Cr-release assay (46). The target cells used were syngeneic cells CT26.WT, βgal-expressing CT26.CL25, and $βgal_{876-884}$ peptide-pulsed CT26.WT as well as a MHC-mismatched EL4 cells (H-2^b) pulsed with $βgal_{497-504}$ peptide. The percentage of specific lysis was calculated from triplicate samples as follows: [experimental lysis – spontaneous lysis / maximum lysis – spontaneous lysis] x 100. The average spontaneous release was 10% to 16% of the total ^{51}Cr incorporated.

Anti-βgal antibodies detection by Enzyme Linked Immunosorbent Assay (ELISA)

Microtitre plates (96 wells, Costar, Cambridge, MA) coated with soluble βgal were used for analysis of anti-βgal antibody (47) in the serum of the immunized mice. The plates were incubated with soluble βgal (400 ng in 50 μl PBS /well, Sigma, St. Louis, MO) at 37° C overnight. The wells were then blocked by incubation with 3% bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 (TPBS) at room temperature for 2h. Sera from immunized mice at various dilutions were incubated in the coated wells for 2 h at room temperature, and the plates were washed 3 times with TPBS. The wells were then incubated with peroxidase labeled goat anti-mouse IgG (1:2000, KPL., Gaithersburg, MD) for 1 h at room temperature. After 3 washes with TPBS, the peroxidase substrate, azino-di(3-ethyl-benzthiazoline sultanate), was added and incubated at room temperature for 15 min. The absorbance was read at 405 nm in microplate reader (Molecular Devices, Menlo Park, CA).

In vivo protection against tumor challenge

Mice were immunized twice 14 days apart as described above. Two weeks after second immunization, mice (n=4 to 6) were challenged by intravenous injection of 5 X 10^5 βgal-expressing CT26.CL25 in 200 μl PBS. This model generates multiple diffuse pulmonary metastases that were highly lethal (48). Two weeks after tumor challenge, the mice were killed, their lungs harvested, fixed in 4% paraformaldehyde, and stained for βgal expression with X-gal (49). The blue stained lung nodules were enumerated in a blinded fashion.

Statistical analysis

The results are expressed as the mean ± standard deviation for each group. The significance of differences among groups was analyzed by one-way or two way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test for multiple comparisons. Before executing the ANOVA, data were tested for normality and equal variance. If any of those tests failed, data were compared using a Kruskal-Wallis one-way ANOVA on ranks. A P value of <0.05 was set for the significance of difference among groups. The statistical analysis was performed with SigmaStat software (Jandel Scientific, San Rafael, CA).

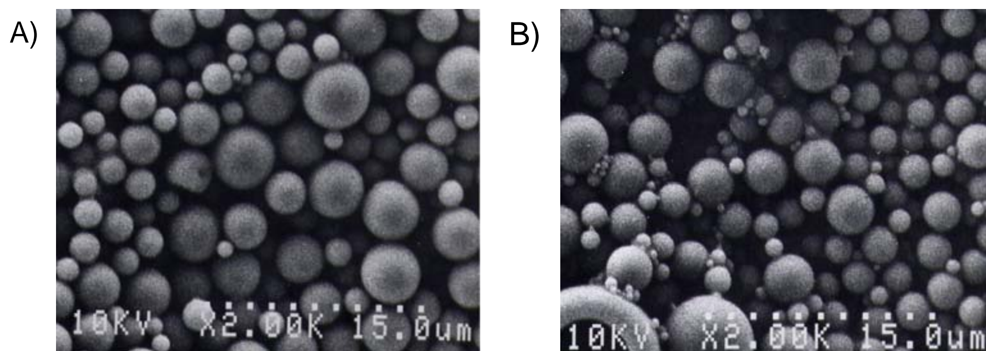

Figure 1: Scanning electron micrographs of PLGA microspheres encapsulated with A) or without B) Ad5-βgal (bar = 15 μm).

RESULTS

Encapsulation of adenovirus in PLGA microspheres: encapsulation efficiency, physical characteristics, and in vitro release

Encapsulation efficiency of adenovirus in PLGA microspheres was 24.8 ± 1.4 % (n=3) as determined by ^{125}I-Ad5-βgal as a tracer. About 70% of the polymer used in the encapsulation was recovered as freeze dried microspheres. Based on these values, viral concentration achieved in a typical formulation was estimated to be 1 X 10^7 PFU of Ad5-βgal/ mg of PLGA microspheres. The scanning electron micrographs showed spherical and compact structure of PLGA microspheres. Their diameter ranged between 0.5 to 5 μm with an average 2.49 ± 0.44 μm (determined by dynamic light scattering; n=7 experiments). The PLGA microspheres containing adenovirus were similar to the control empty microspheres both in size and morphology (Figure 1).

The release of the encapsulated virus from the PLGA microspheres was estimated using ^{125}I-Ad5-βgal. Approximately 15 % of the entrapped viruses were released *in vitro* from PLGA microspheres over 11 days, half of which occurred within the first 24 h (Figure 2). In separate experiments the functional integrity of the encapsulated virus was assessed in an infectivity assay using permissive 293 cells. PLGA microspheres containing the encapsulated Ad5-βgal were incubated with 293 cells for 12 days and functional virus was quantified as PFU. Assuming that the PFU values reflect the infectious viral particles available within the first 5 days, about 11.4 ± 3.6 % of the encapsulated virus retained the functional activity.

PLGA microsphere delivery of Ad5-βgal to APCs in vitro

PLGA microsphere delivery of Ad5-βgal to professional APCs *in vitro* through phagocytosis was evaluated using a murine macrophage cell line (J774A.1). J774A.1 cells were co-cultured with PLGA microspheres containing ^{125}I-Ad5-βgal and the viral uptake was quantified based on radioactivity. The cellular uptake of the encapsulated viral particles was about 7 times higher than that of free virus for 24 h incubation (Figure 3). The resulting expression of the βgal reporter gene was also higher for the encapsulated Ad5-βgal in comparison with the free virus (ANOVA, $P<0.0001$). About 3.1 % of J774A.1 cells were transduced 6 days after co-culture with 250 μg of PLGA microspheres containing 5 X 10^6 PFU Ad5-βgal, whereas only 0.2% of the cells were transduced by direct infection with 5 X 10^6 PFU Ad5-βgal alone for the same period (Figure 4A). A preliminary study also showed that PLGA microsphere delivery of Ad5-βgal over a period of 6 days results in transduction of 10.3 % of primary human DCs cultured in vitro (Figure 4B).

Effect of encapsulation on viral dissemination in vivo

The biodistribution of Ad5-βgal changed markedly when the viruses were administrated subcutanously to mice in the encapsulated state as compared to the free state. About 60.9 % and 24.7 % of the total injected ^{125}I-Ad5-βgal encapsulated in PLGA microspheres were retained at injection site at day 1 and day 5 respectively, whereas only 8.3 % and 3.1% of injected free viruses remained at the injection site at the same time points (Figure 5A). The viral uptake in various organs was also

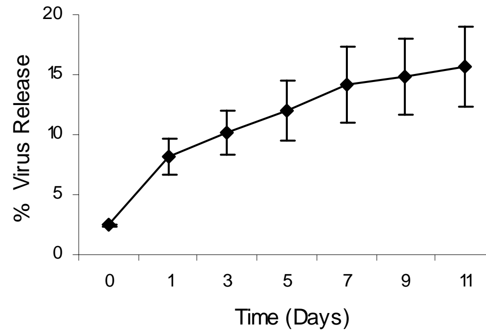

Figure 2: Release of ^{125}I-Ad5-βgal encapsulated in PLGA microspheres *in vitro*. PLGA microspheres (5 mg) containing ^{125}I-Ad5-βgal (0.2 Ci) were suspended in PBS (1 ml) and shaken in a water bath at 37 °C. At each time point, the supernatant containing released virus was collected by centrifugation. The amount of virus released from microspheres was determined by measuring radioactivity (CPM) in supernatant and pellet (microspheres). The percentage of release was calculated as follows: $[CPM_{supernatant} / CPM_{pellet} + CPM_{supernatant}]$ X 100.

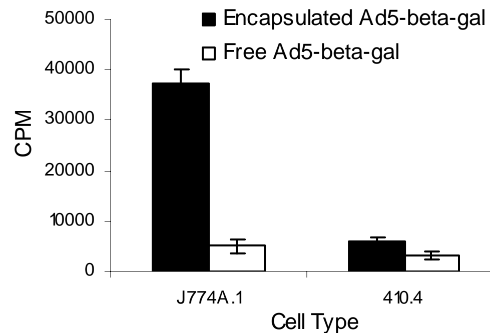

Figure 3: PLGA microsphere delivery of Ad5-βgal to macrophage cells. ^{125}I-Ad5-βgal encapsulated in PLGA microspheres or same amount of free ^{125}I -Ad5-βgal were mixed with murine macrophage cells (J774A.1) or murine mammary carcinoma cells (410.4) and incubated for 24 h at 37 °C. The free microspheres were completely removed by washing with PBS for 5 times. The amount of ^{125}I - Ad5-βgal that entered cells was determined by measuring the CPM in the cell lysate. Error bars indicated SD (n=3).

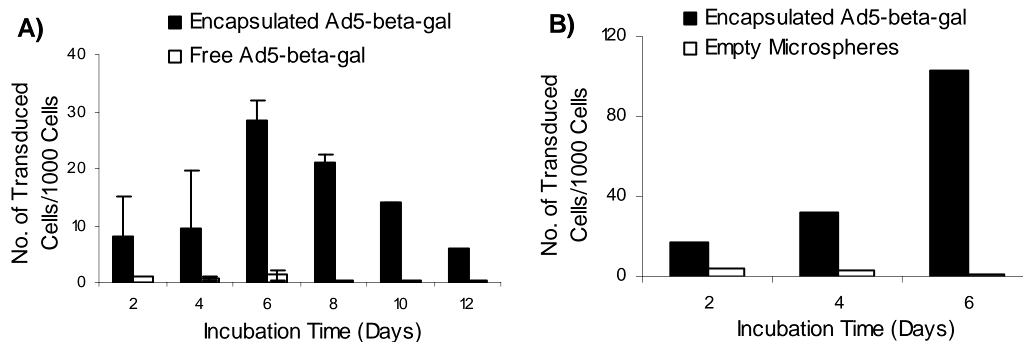

Figure 4: The transgene (βgal) expression in APCs after PLGA microsphere delivery of Ad5-βgal. PLGA microspheres (250 μg) containing Ad5-βgal (2.5 X 10^6 PFU) was co-cultured with human DCs or murine macrophage cells (J774A.1) for different periods. Same amount of free Ad5-βgal or empty PLGA microspheres were used as controls. A) βgal expression in J774A.1 cells. B) βgal expression in human DCs.

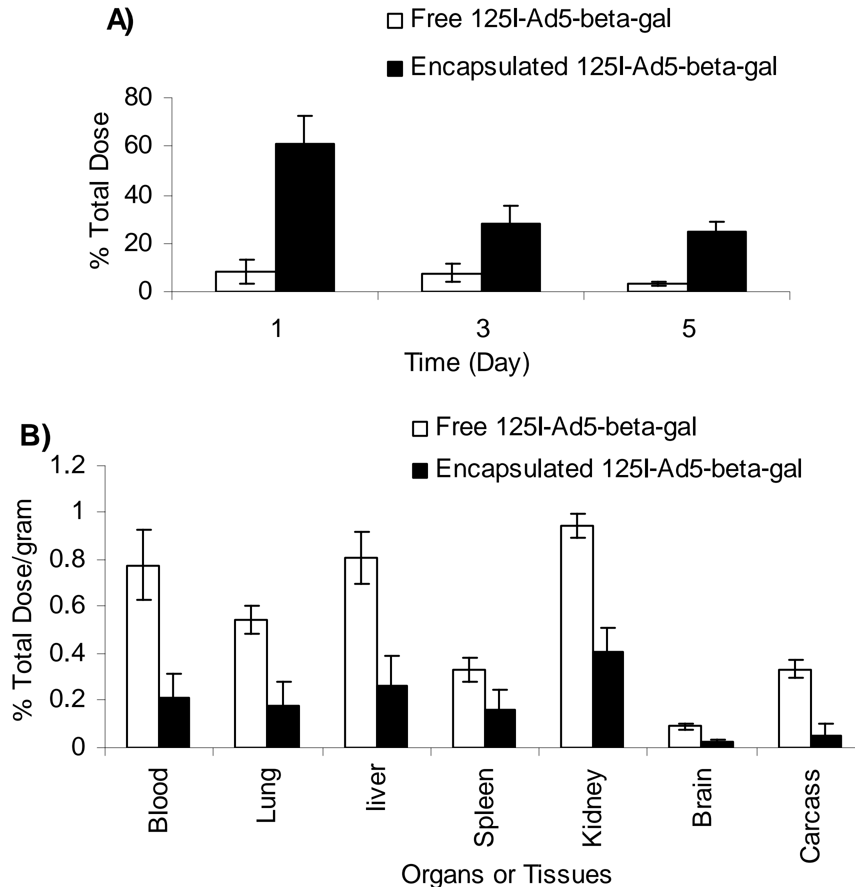

Figure 5: Biodistribution of ^{125}I -Ad5-βgal encapsulated in PLGA microspheres after subcutaneous (s.c.) administration. C57BL/6 mice (n=3) were s.c. injected with encapsulated or free ^{125}I -Ad5-βgal (1.2 µCi; 2 X 10^7 viral particles). Mice were sacrificed 1, 3 or 5 days after injection, and radioactivity (CPM) in tissues were determined A) % total injected dose remaining at the local injection site B) % injected dose/g in each organ or tissue over a period of 7 days.

significantly lower for encapsulated Ad5-βgal in comparison with the free virus over a period of 7 days (Figure 5B) (ANOVA, P<0.0001).

Induction of cytotoxic T cell responses

Based on the observation that professional APCs can ingest and express of Ad5-βgal delivered by PLGA microsphere, we further analyzed whether this delivery system could be used to elicit CTL response to the βgal encoded by Ad5-βgal *in vivo*. Significant CTL responses against a βgal-transfected syngeneic tumor line (CT26.CL25: H-2^d) or βgal peptide-pulsed CT26.WT cells were found in mice immunized with Ad5-βgal encapsulated in PLGA microspheres. The CTL activity in the mice immunized by microspheres containing 1 X 10^8 PFU encapsulated Ad5-βgal reached similar level to that immunized with same amount of free Ad5-βgal (Figure 6A and B) (ANOVA followed by Student-Newman-Keuls post hoc test, P>0.05). However the infectivity assay showed that only 1.1 X 10^7 viral particles encapsulated inside PLGA microspheres were viable. These results indicate that CTL induction by the encapsulated Ad5-βgal could be achieved at about 10 fold lower concentration of viable virus when compared with free Ad5-βgal. The CTL was MHC restricted, since EL4 (H-2^b) cells pulsed with H-2^b–binding βgal peptide were not killed. Splenocytes isolated from control mice immunized with empty PLGA microspheres and stimulated *in vitro* by peptide-pulsed syngeneic splenocytes did not exhibit any CTL activity (Figure 6C).

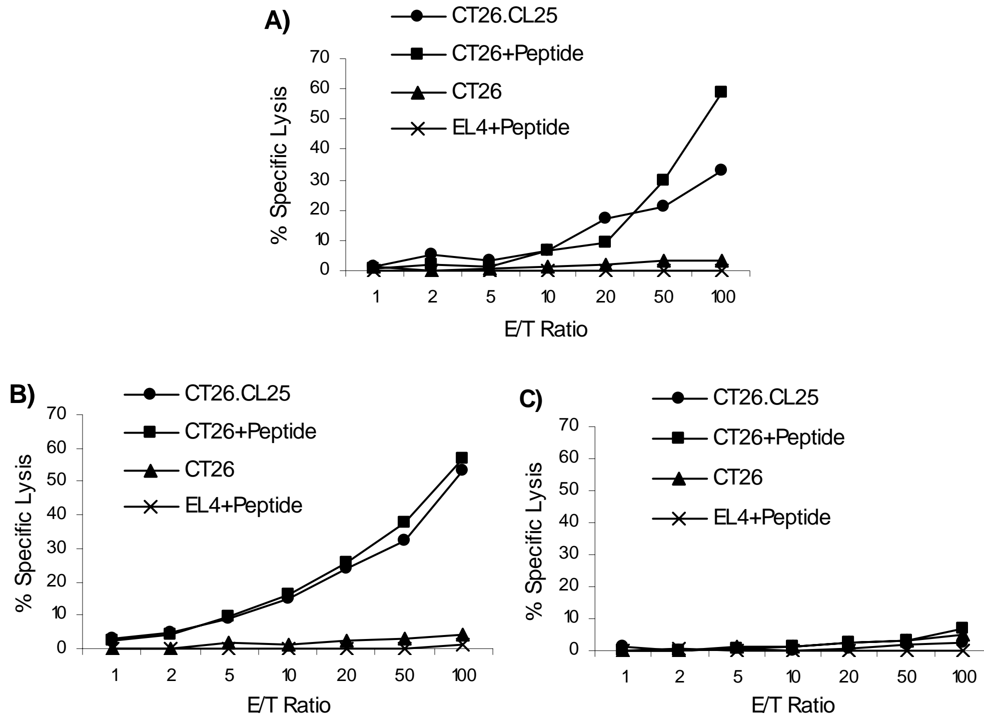

Figure 6: CTL responses against βgal in BABL/c mice immunized with Ad5-βgal encapsulated in PLGA microspheres. Mice (n=4) were immunized by s.c. injection twice, 14 days apart, with 10 mg of PLGA microspheres containing 1 X 10^8 PFU Ad5-βgal (A), 1 X 10^8 free Ad5-βgal (B) or 10 mg empty microspheres (C). Two weeks after the second immunization, splenocytes were harvested, stimulated for 5 days *in vitro* with syngeneic splenocytes pulsed with a βgal peptide, and assayed for cytotoxicity (^{51}Cr-release assay) against three syngeneic colon carcinoma target cells (parental CT26.WT cells, βgal-expressing CT26.CL25 cells, and $βgal_{876-884}$ peptide-pulsed CT26.WT cells) as well as the $βgal_{497-504}$ peptide pulsed allogeneic EL4 cells.

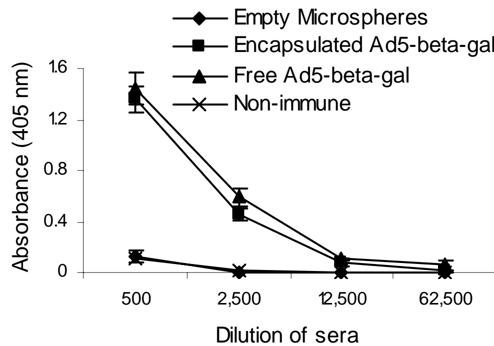

Figure 7: Antibody responses (IgG) to transgene product (βgal) in mice immunized with Ad5-βgal encapsulated in PLGA microspheres. Balb/c mice were vaccinated as described in Fig. 6. Sera collected 2 weeks after second immunization were tested on soluble βgal protein coated plates (400 ng/well) by ELISA**.**

Induction of humoral responses
The sera from immunized mice were assayed for anti-βgal antibodies by ELISA. Ad5-βgal encapsulated in PLGA microspheres induced strong IgG responses against βgal, which was comparable to that induced by free Ad5-βgal (Figure 7).

In vivo protection against tumor challenge
Since PLGA microsphere delivery of encapsulated Ad5-βgal could induce strong humoral and CTL responses with reduced viral dissemination, we further tested the effect of encapsulated Ad5-βgal immunization on protection against a challengewith βgal-expressing tumor cells, CT26.CL25. Results showed that control mice immunized with empty PLGA microspheres had pulmonary metastases that were too numerous to count, whereas mice immunized with either PLGA microsphere encapsulated or free Ad5-βgal induced a significant reduction in CT26.CL25 pulmonary metastases (Figure 8) (ANOVA, $P<0.0001$). The visual appearance of the lungs following to immunization with the formulations has been shown in Figure 9.

DISCUSSION

Replication-deficient adenoviral vectors have demonstrated great potential as vaccine vectors for the transfer of the genes encoding pathogen or cancer-associated antigens to induce protective antigen-specific immune responses. However, researchers are facing the challenges associated with tissue-specific targeting of vectors and the vector-mediated immunogenicity. Development of a specific and efficient technique for the *in vivo* delivery of adenoviral vectors to APCs in particular DCs as a central element in the development of antigen-specific immune responses is expected to overcome problems associated with viral dissemination and undesired immune responses. In this report, we have evaluated the use of PLGA microspheres for the delivery of recombinant adenovirus to professional APCs as a novel vaccine delivery strategy. We reasoned that this approach would reduce the viral dissemination *in vivo* without compromising the induction of immune responses.

Figure 8: Protection against lethal tumor challenge in mice vaccinated with Ad5-βgal encapsulated in PLGA microspheres. Balb/c mice (n=4 to 6) were immunized as described in Figure 6. Two weeks after second immunization, mice were challenged by i.v injection of 5 X 10^5 βgal expressing CT26.CL25 cells. After another 13 days they were sacrificed and their lungs harvested, fixed, and stained for βgal activity. Surface blue-staining (βgal^+) metastatic lung nodules were counted. Only metastatic nodules < 250 could be reliably counted; lungs with > 250 metastatic nodules were assigned an empirical number of 250.

Figure 9: Photographs of the lungs from the mice immunized with A) empty PLGA microspheres B) Ad5-βgal encapsulated in PLGA microspheres C) free Ad5-βgal; as described in Figure 8.

Our results show that the virus can be encapsulated in PLGA microspheres with significant retention of biological activity. A final biological activity of about 11% is encouraging in view of the shearing and denaturing effects on the virus during the encapsulation using double emulsion technique. It is worth noting that most formulation or preparative procedures would results in substantial loss of biological activity of the adenoviruses. For example, only 5 % of viruses are infectious after standard CsCl gradient purification (50). In order to minimize the shearing, vortexing rather than sonication was used for both steps of emulsification. It may be possible to increase the biological activity by co-encapsulation of the virus with proteins such as bovine or human serum albumin. The loss of infectivity may at least in part be due to the denaturation of viral surface proteins mediating viral binding to the host cells. Since PLGA microspheres deliver their encapsulated viruses to APCs through phagocytosis rather than direct binding with cell receptors, loss of functional activity of the surface proteins is unlikely to affect the expression of the transgene by the APCs. Thus some of the Ad5-βgal that lost its natural infectivity during formulation might be still infectious once within the endosomes.

One important characteristic of professional APCs such as macrophages and DCs is their capacity for phagocytosis of particulate antigens below 10 μm. We prepared PLGA microspheres with a particle size below 10 μm (range: 0.5 to 5 μm) to deliver the encapsulated virus to APCs through phagocytosis. Using ^{125}I- labeled virus, we showed that encapsulated Ad5-βgal entered J774A.1 macrophage cells more efficiently through phagocytosis (7.4 times higher) than free viruses did through natural infection process using cell surface receptors. Before the transgene expression the encapsulated virus needs to be released first from the polymer, and then released from endosomes to cytoplasm, and enter the nucleus of the host cells. The release of macromolecules such as proteins or plasmid DNA from biodegradable PLGA microspheres depends on the molecular weight of polymer (29, 51). We have used low molecular weight PLGA (6,000 g/mole) for encapsulation since it releases encapsulated macromolecules at a faster rate than the high molecular weight polymer. Although our *in vitro* data indicate slow release (15% in 11 days) of the virus from the polymer, the rate of release in the endosomes *in vivo* would be faster due to acid catalyzed hydrolysis of PLGA. By X-gal staining assay, we are able to show that 10.3 % human DCs and 3.1% J774A.1 macrophage cells expressed Ad5 encoded βgal after phagocytosis of PLGA microspheres containing encapsulated Ad5-βgal. Further, the transduction efficiency of the encapsulated virus in J774A.1 cells was significantly higher than that of free virus through natural infection. Timares et al (17) have proposed that a single injection of 500–1,000 transfected DCs can produce a response comparable to that of a genetic immunization with gene gun. According their calculation, even if only 0.5-1% of Langerhans cells, a resident DC population in skin, are transfected after gene gun immunization (shooting area ≈1 cm^2), it is sufficient to initiate the immune response to transgene protein (Langerhans cells density in skin ≈1,000 cells/ mm^2) (52). Based their data, it is reasonable to expect that the encapsulated Ad5-βgal, which transduce 10.3 % of DCs *in vitro* would transduce enough Langerhans cells *in vivo* for initiation of T cell responses.

The above results are consistent with the *in vivo* immune responses and immune protection effects against a lethal challenge with βgal tranfected tumor. The magnitude of humoral and cytotoxic immune responses elicited by the PLGA microsphere encapsulated virus was similar to the free virus at comparable total viral dose. However the encapsulated formulation only had about 11% retention of infectious activity of the virus.

Therefore the immune responses shown were achieved by about 1/10th of the dose of infectious virus in the encapsulated form as compared to the free virus. Similarly, immune protection against a lethal tumor challenge was also achieved with a 10 fold lower dose of the infectious virus in encapsulated form as compared to the free virus. Thus PLGA microsphere delivery may achieve protective immune responses at a significantly lower infectious dose in comparison with the free virus however further dose response studies are needed to test this hypothesis .

Beer et al reported encapsulation of adenovirus in PLGA microspheres of an average size of 100 μm, for viral delivery to glioblatoma through intratumor injection. These microspheres were designed to avoid phagocytosis of by APCs and to serve as a slow release delivery system for gene therapy. Their results showed a reduced induction of neutralizing antibodies, which would permit effective repeated administration of the virus (45). In our study, PLGA microspheres of small size (average size of 2.45 μm) were designed for phagocytosis by APC for enhanced immune response. These results indicate that by controlling the particle size of PLGA microspheres, immunogenicity of the encapsulated adenovirus can be reduced or enhanced.

The viral biodistribution was markedly changed by PLGA microsphere delivery of the encapsulated Ad5-βgal through s.c. administration *in vivo*. In the case of encapsulated virus, significantly higher percentage of virus remained in local injected site, and the viral dissemination to organs and tissues were significantly decreased. Ad5-βgal inside microspheres may be eliminated from the subcutaneous site of injection in several ways. First, they may be phagocytosed by Langerhans cells, which serve as resident skin DCs. Second, they may enter local lymphatic circulation and be drained to the nearest lymph nodes. Third, about 5 % or so virus may be released from microspheres in first few hours of administration before phagocytosis by Langerhans cells. This released virus possibly will behave same as free virus. These different routes of clearance may account for changes in the organ distribution when Ad5-βgal is administered in the encapsulated form. The retention of Ad5-βgal in local injected site and the decrease of viral distribution to organs and tissues can reduce the systemic toxicity caused by virus dissemination. Decreased dissemination to various organs including liver would be advantageous in limiting the side effects of adenovirus immunization such as adenovirus induced hepatitis (53, 54). Our result is consistent with the recent study showing that intramuscular injection of adenovirus encapsulated in PLGA microparticles in immunocompetent mice prolong transgene (β-galactosidase) expression in the muscles injected with adenovirus-loaded PLGA microparticles as compared in control muscles injected with purified adenovirus stocks (55). The retention of virus in the site of injection is expected to induce immune response for longer period of time. The major concern with the method used for the study of virus dissemination in this work is the degradation of viral proteins labeled with ^{125}I after viral up take by the cells. In the applied iodination method, most of the ^{125}I label is confined to hexons and fibers in viral structure (40). Based on the N-end rule pathway of protein degradation *in vivo,* hexons and fibers are classified as long-lived proteins with half life >30 h so they would stay in the infected cells long enough to be monitored for a few days post injection of the viral particles (56, 57). Even so further studies using an alternative method such as analyzing β-galactosidase reporter gene expression are needed to confirm the presented data on viral dissemination *in vivo*.

To our knowledge this is the first report of PLGA microsphere delivery of adenovirus to professional APCs for protective immune responses against viral encoded transgene. In conclusion, we have demonstrated that replication-deficient adenovirus vectors can be encapsulated into biodegradable small PLGA microspheres (< 5 μm) with retention of biological activity. Such microspheres with Ad5-βgal can enhance the delivery of virus to APCs through phagocytosis resulting in increased transgene expression in these cells *in vitro* and achieve protective immune responses *in vivo* at a significantly lower infectious viral dose as compared to the free virus. We have also shown that PLGA microsphere delivery of Ad5-βgal for subcutaneous immunizations results in substantially decreased viral dissemination in organs including liver.

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