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Genetic immunization with Lung-Targeting Macroaggregated Polyethyleneimine-Albumin Conjugates Elicits Combined Systemic and Mucosal Immune Responses

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Genetic immunization is a novel form of vaccination in which transgenes are delivered into the host to produce the foreign protein within host cells. Although systemic immune responses have been relatively easy to induce by genetic immunization, the induction of regional and mucosal immunity has often been more challenging. To address the problem of eliciting mucosal immunity in the lung, we utilized macroaggregated albumin to target plasmid DNA to the lung. Macroaggregated albumin is trapped in the lung after i.v. injection, and it is routinely used in radiolabeled form as an imaging modality to evaluate pulmonary blood flow. To couple DNA to this targeting agent, polyethyleneimine (a polycation that binds DNA and enhances transfection) was conjugated to serum albumin, and the conjugate was aggregated by heating to produce particles of 25–100 µm. The resulting particles bound plasmid DNA avidly, and when injected i.v. in mice, the particles distributed in the peripheral lung tissue in the alveolar interstitium. Particle-bound luciferase plasmid transfected a variety of cell lines in vitro, and after i.v. injection, gene expression was detected exclusively in the lung. Using human growth hormone as the encoded foreign Ag for immunization, i.v. injection of the particle-bound plasmid elicited both pulmonary mucosal and systemic immune responses, whereas naked DNA injected either i.v. or i.m. elicited only systemic responses. Thus, particle-bound plasmid DNA may have utility for genetic immunization by intra-vascular delivery to the lung and potentially to other organs and tissues. The Journal of Immunology, 2000, 164: 6313–6321.

Gene therapy and genetic immunization using plasmid expression vectors are being developed to utilize modern advances in molecular biology for the treatment and prevention of human disease. Transfer of expression vectors into cells and tissues has had enormous experimental importance for a number of years, and its value in gene therapy and genetic immunization is now being evaluated at the clinical level. The essential requirement for both techniques is the delivery and expression of the desired sequences in the appropriate tissues. Viral vectors are very efficient at delivery and expression, but inflammatory and immunologic responses induced by the virus proteins limit their utility for repeated administration (1–3). Numerous methods of nonviral delivery for in vivo transfection have been studied, including gene gun administration in skin and mucosal tissues (4–7), injection of naked DNA or DNA complexed with binding agents directly into the desired tissue by needle (8–10) or jet devices (11–13), aerosol or intranasal instillation for delivery to the airway (14–18), i.v. injection of DNA complexed with poly aggregates or in liposomes (19, 20), and oral particle administration (21). Application of these methods for genetic immunization has been recently reviewed (22).

Although systemic immune responses are readily elicited by genetic immunization, mucosal immune responses are more difficult to achieve in general (23), except with live attenuated vaccines like the polio virus vaccine. Eliciting mucosal immunity with genetic vaccines has also been more difficult either with the widely used gene gun or with naked DNA inoculation (24, 25). The appreciation of the overlap between the tissues of the common mucosal immune system (26, 27) has resulted in experiments that have administered plasmid to mucosal tissues by a variety of routes. Genetic immunization via intranasal administration of viral vectors (14) or DNA-liposome complexes (16), and intravaginal injection of plasmid via gene gun (6) have succeeded in inducing mucosal responses in the female genital tract and elsewhere (28).

To elicit mucosal responses to genetic immunization in the lung, we targeted pulmonary interstitial macrophages and dendritic cells utilizing macroaggregated albumin (MAA), a safe and readily prepared clinical agent that has been used in nuclear medicine for many years to image pulmonary blood flow (29). These particles are of a sufficient size (20–50 microns) that when injected i.v., they cannot pass readily through the pulmonary capillary bed (30, 31); hence, they accumulate in peripheral lung tissue (where the particles enter the interstitium for eventual clearance by interstitial macrophages) (30, 32). When the particles are radiolabeled, lung perfusion can be imaged because the particles collect in the pulmonary capillary bed in proportion to blood flow (33). To take advantage of this property for vaccination purposes, we conjugated serum albumin and polyethyleneimine (PEI; a polycation that

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3 Abbreviations used in this paper: MAA, macroaggregated albumin; PEI, polyethyleneimine; HSA, human serum albumin; p, plasmid; sGH, human growth hormone; GFP, green fluorescent protein; BALF, bronchoalveolar lavage fluid; UB, ubiquitin.
binds DNA and can enhance transfection (20) and then aggre-
gated the conjugate by heating to produce particles 25–100 μm in diameter. These MAA-PEI particles bound plasmid DNA avidly, as shown by the presence of stained DNA on the particle surfaces and also by the rapid removal of DNA from solution in the pres-
ence of the particles. We report that in addition to transfecting a variety of cell lines in vitro, i.v. injected MAA-PEI particles loaded with plasmid DNA selectively transfect the lung tissue of mice. In contrast to the systemic immunization elicited by i.m. or i.v. injection of naked DNA, the expressed Ag delivered to the
lungs by MAA-PEI particles effectively elicited high-level mucosal
as well as systemic immunity.

Materials and Methods

Preparation of particles
A stirred solution of human serum albumin (HSA; 50 mg, 0.72 μmol in 1 ml 0.1 M NaHCO₃, pH 8.5) was reacted with 0.25 mg (0.8 μmol) N-
succinimidyl 3-(pyridyldithio)propionate (Pierce, Rockford, IL) in 10 μl DMSO. A stirred solution of PEI (m.w., 25,000; Aldrich, Milwaukee, WI) was prepared (18 mg, 0.72 μmol in 1 ml H₂O), the pH was adjusted to 8.5 with 1 M HCl, and N-succinimidyl 3-(pyridyldithio)propionate (0.25 mg, 0.8
μmol in 10 μl DMSO) was added with stirring. When desired, fluorescein isothiocyanate (1 mg in 10 μl DMSO) was added at this point. After sev-
eral hours, the HSA and PEI solutions were put separately over NAP-10 columns (Pharmacia, Piscataway, NJ) equilibrated with PBS. The PEI el-
uate was treated with 15 μg Reductacryl (Calbiochem, San Diego, CA) for
several hours and then filtered slowly into the stirred HSA solution to a
final 1:2 mole ratio of PEI to HSA. Stirring was continued overnight,
the solution made up to 5 ml with PBS, and the pH was adjusted to 5.5–6.0
with 1 M HCl. Aggregation was accomplished at 85°C with vigorous stir-
ing. The MAA-PEI aggregates were gently centrifuged, rinsed twice by
resuspending and centrifuging in PBS, and resuspended in a final volume
of 4 ml PBS. The particles were used at a 10% v/v dilution (unless oth-
erwise specified), thereby having a maximum of 1.25 μg/μl HSA in the
working suspensions.

Particle size analysis and DNA binding assessment
MAA-PEI-DNA particle size was determined by analyzing the formula-
tions dispersed in aqueous media with dynamic light scattering using a
Nanophor 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Bar-
abara, CA). Particle size was also verified by light microscopy.
DNA binding was assessed microscopically by allowing ethidium bro-
mide to bind the plasmid before incubation with the particles. Ethidium bromide (1 μl of a 100 μg/ml solution) was added to 10 μg of plasmid
DNA in a final volume of 10 μl. After 10 min, the solution was diluted 1:10 in PBS, and 10 μl (containing 1 μg of plasmid) was added to 40 μl of a
1.10 dilution of MAA-PEI stock solution. After incubation at room tem-
perature for 20 min, an aliquot of the particle suspension was placed on a
slide and photographed under UV illumination. Solutions of ethidium bromide similarly diluted did not stain the MAA-PEI particles significantly, as
seen by fluorescent microscopy.

To assess DNA binding by removal of plasmid from solution, 250 ng of
plasmid in 5 μl PBS was added to a series of tubes containing increasing
concentrations of stock MAA-PEI particles or un conjugated MAA parti-
cles in 10 μl PBS. After 20 min incubation, the samples were centrifuged
(3 min at 5000 × g), and the supernatant aliquots were added to a sucrose
loading buffer, pipetted into wells of a 0.6% agarose gel, and electropho-
resed in Tris-acetate buffer at 10 V/cm for 1 h. The gel was stained with
ethidium bromide and photographed with UV transillumination.

Plasmids
The luciferase plasmid pGL3 (Promega, Madison, WI), into which the
CMV enhancer/promoter region and human growth hormone (hGH) poly-
adenylation sequence from CMV 1 (34) were inserted, and the green flu-
escent protein (GFP) plasmid pEGFP (Clontech, Palo Alto, CA) were
used as reporter genes for transfection. The plasmids used for immuniza-
tion studies included pCMV-hGH encoding the hGH gene and pCMV-
UB23, which encodes and expresses a fragment of the HIV gp120 envel-
lope protein (a kind gift of Kathryn Sykes, University of Texas Health
Science Center, Dallas, TX) which had been fused to ubiquitin (35–38).
Because the fusion product for the latter plasmid contains the ubiquitin protein, the product is directed to proteasomes that degrade the protein into
peptides for major histocompatibility class I presentation (39), thus elic-
ting CTL. Plasmids were prepared using reagents and columns for endo-
toxin-free DNA (Qiagen, Valencia, CA) and then were dissolved in endo-
toxin-free water at the desired concentration.

In vitro studies
Tested cell lines included RAW 264.7 (monocyte/macrophage), Jurkat
(T cell), Raji (human Burkitt lymphoma), U937 (human monoblastoid)
cells, P815 (murine mastocytoma), A549 (murine lung carcinoma), HEPA-1
(murine hepatoma), P815 (murine mastocytoma), D451 (murine fibro-
blast), CIC12 (murine myoblast), and 293 (human epidermal kidney cell).
Cells (100,000/well) were cultured in a 24-well format in 1 ml RPMI 1640
medium (Life Technologies, Grand Island, NY) with 10% FCS and 20
μg/ml ciprofloxacin (Miles Pharmaceuticals, West Haven, CT) at 37°C in
a 5% CO₂ atmosphere and were preincubated for 24 h (unless otherwise
specified) before addition of transfecting agents. To bind plasmids to
MAA-PEI, the particles were diluted in PBS, and then appropriate con-
centrations of plasmid in PBS (usually 1 μg/40 μl of particle suspension)
were added to the suspension during gentle vortexing. After a 20-min incu-
bation at room temperature, the particle complexes were added to the
cultures with swirling to distribute the particles evenly. The cell cultures
were incubated for 24 h before assay for luciferase or GFP expression.
Comparison transfection methods included complexing the plasmid with
Lipofectamine (Life Technologies) in Optitrend (Life Technologies) or
with PEI alone in PBS according to standard protocols (20).

Luciferase assay
The culture medium was removed from the cells and 1 ml of lysis buffer
(Promega) was added to each well. After gentle agitation on a rotary plat-
form for 15 min at room temperature, aliquots of each culture lystate were
assayed by mixing 10 μl of the lysate with 50 μl of luciferin substrate and
measuring light output for 15 s in a Turner TD-20C Luminoimeter. For
luciferase study data presentation, the response was converted to ng of
luciferase/well, with 1 ng producing 10,000 lumens in the Turner lumi-
oimeter under these assay conditions.

GFP detection
Cells in which GFP has been expressed from the pEgFP plasmid were
examined by UV microscopy at appropriate time points after culture ini-
tiation (usually after 24 h). Thick tissues from in vivo studies were em-
bedded in freezing medium (OCT compound, Miles, Elkhart, IN), and
frozen sections were examined by light and UV microscopy.

In vivo studies
All animal experiments were approved by the Institutional Review Board
for Animal Studies (Baylor College of Medicine), BALB/c mice (male and
female; Harlan Sprague-Dawley, Houston, TX) were anesthetized with
Avertin (0.5 ml/25 g mouse) and injected i.v. with MAA-PEI particles via
the tail vein, which delivers injected materials predominantly to the lung
(30, 40 – 43). Particle/DNA (5 μg DNA) suspensions in PBS were injected
as a single 200-μl volume in the tail vein. Mice were sacrificed at the
desired time points by lethal anesthesia, and tissues were harvested for
analysis by standard techniques. For particle localization, the mice were
sacrificed 30 min after injection by lethal anesthesia and exsanguination
by abdominal aorta transection, and the lungs were harvested, inflated with
OCT compound, frozen, and sectioned. For luciferase gene expression, the
mice were sacrificed at 48 h, and the tissues of interest were harvested,
placed in 1 ml of luciferase lysis buffer (Promega), and homogenized using
a conical ground glass tissue grinder (Kontes Duall 23; Fisher Scientific,
Houston, TX). A 10-μl aliquot of the homogenate was added to 50 μl of luciferase substrate, light output was measured, and the data were con-
verted to ng/organ for presentation as described above.

Humoral immunization studies
Groups of five mice were exposed to pCMV-hGH or pCMV-UB-HIV us-
ing particles loaded with 5 μg of DNA and injected via tail vein in a single
200-μl volume of PBS. Control groups included un injected, 50 μg naked
plasmid DNA in 50 μl PBS injected i.m., 5 μg naked plasmid DNA in 200 μl
PBS injected i.v., and 5 μg of plasmid DNA complexed with 180 nmol
PEI in 200 μl PBS injected i.v. Sequential serum samples were obtained at
biweekly intervals by tail bleed. Sera were stored frozen (at −20°C) until
they were assayed. Vaginal secretion samples were obtained by the
wet mount technique (wet mount after freezing), using 60 μl of PBS with
wetting solution. Samples were collected at each time point after
immunization, in addition to blood collection, bronchoalveolar lavage
was performed to collect pulmonary surface secretions. Bronchoalveolar
lavage fluid (BALF) was obtained from both untreated and immunized

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FIGURE 1. Macroparticulated PEI particles and DNA binding. PEI-conjugated albumin was aggregated as described in Materials and Methods. The particles are shown with bright field illumination (×400 original magnification; A). Plasmid DNA was stained with ethidium bromide, diluted, and then incubated with the MAA-PEI. UV illumination (B) demonstrates intense ethidium bromide fluorescence of the particle-bound DNA (×400 original magnification).

mice sacrificed by Avertin anesthesia and exsanguination via cardiac puncture. The trachea was surgically exposed and cannulated with PE50 tubing (outer diameter, 0.965 mm; Clay Adams, Sparks, MD), through which a total volume of 1.0 ml PBS was slowly injected and aspirated three times, with typically 80% of the lavage fluid volume recovered.

ELISA
Serum and BALF were assayed by ELISA for total and isotype-specific Ab to human growth hormone (purified hGH protein was obtained from Calbiochem). The desired Ag was coated onto microtiter plates (Immunolon II; Dynex Technologies, Chantilly, VA) at 0.5 μg/ml (50 ng/well) in PBS buffer (pH 7.3) overnight at 4°C. This loading quantity was chosen as the most cost effective by comparison of various concentrations with serial dilutions of a positive control antiserum. The wells were then blocked with 5% nonfat milk in PBS. Sera and lung lavage fluids were diluted in PBS, and aliquots added to wells were incubated overnight at 4°C. Sera and lavage fluids from age-matched, unimmunized BALB/c mice served as negative controls in each assay. After five washes with PBS-Tween (0.1%), bound Abs were detected with HRP-conjugated goat or rabbit anti-mouse Ig (Bio-Rad, Hercules, CA) or anti-mouse IgA, IgM (Sigma), IgG1, IgG2a, or IgG3 (Serotec, Raleigh, NC) diluted in PBS-Tween. Reactions were developed using TMB substrate (Calbiochem), and OD was measured at 405 nm with an SLT microplate reader (TELAC, Research Triangle Park, NC) with a maximal OD for linear reading of 1.4, with the background subtracted. Total Ag concentrations were determined by standard ELISA curves of bound Ag for each isotype.

Cytotoxicity assay
Five mice were exposed to pCMV-UB23 using particles loaded with 5 μg of DNA and injected via tail vein in 200 μl of PBS. Single-cell suspensions were prepared from mouse spleen by mechanical disruption. P815 target cells were loaded with the p18 peptide (RIGQGPGRAFVTIGK) by incubation at 37°C for 1 h at 1 μM. Splenocytes (variable numbers) and targets (10,000 cells/well) were cocultured at desired E:T ratios, and control cultures were prepared from mouse spleen or latex beads. After a 4-h incubation, supernatant aliquots were harvested, stained with FITC conjugated to mouse anti-CD8, washed, and fixed in 1% formalin. Fluorescence was measured on a FACSort (Becton Dickinson, Mountain View, CA) with Lysis II software (Becton Dickinson) to determine the mean fluorescence of sepcific targets. Values were compared using Student’s t test.

Transfection of cell lines by MAA-PEI-bound plasmid
To evaluate transient transfection mediated by MAA-PEI, we compared the plasmid-bound particle transfection to that of Lipofectamine in the presence and absence of serum using a plasmid with the luciferase reporter gene under CMV enhancer/promoter regulation and a monocyte/macrophage lineage murine cell line (RAW264.7) as the transfection target. In this experiment, we used RPMI 1640 culture medium with and without 10% FCS. The level of expression in serum-free medium (Fig. 3) was somewhat better using Lipofectamine than it was with MAA-PEI. However, in the presence of serum, transfection using Lipofectamine was reduced to 2% of that in the absence of serum. In contrast, transfection using MAA-PEI in the presence of serum was three times higher than in its absence and 100-fold greater than that of Lipofectamine in the presence of serum. Negative controls included untreated cells, cells exposed to plasmid alone, and cells exposed to MAA-PEI alone, where there was no detectable luciferase activity. In subsequent experiments, a rough correlation of the proportion of cells transfected and the total luciferase activity in cultures (Fig. 3) was found. Cultures transfected via MAA-PEI with GFP expressing plasmid had ~24 and 15% positive cells in the presence and absence of serum, respectively. Lipofectamine-treated cultures were examined for DNA binding in vitro. Size was determined as described in Materials and Methods, showing particles generally in the range of 25–100 μm in diameter. To demonstrate DNA binding, plasmids were incubated with ethidium bromide, diluted, and added to the particles as described in Materials and Methods. Fig. 1B shows that the particles had bound the ethidium bromide-stained plasmid and were brightly fluorescent, whereas similar low concentrations of ethidium bromide added to the particles in the absence of DNA showed minimal particle fluorescence. To demonstrate quantitative binding, increasing amounts of MAA-PEI suspension or MAA suspension without PEI were added to a fixed quantity of plasmid. After a 20-min incubation, the particles were sedimented by centrifugation, and an aliquot of the supernatant was examined for free DNA by agarose gel electrophoresis. As shown in Fig. 2, essentially complete removal of DNA from solution in the presence of MAA-PEI occurred in the ratios used for cell culture and in vivo experiments (lane with 1.5 μl of stock particles added). MAA not conjugated to PEI did not bind plasmid at all (bottom three lanes).

Results
Particle size and DNA binding
MAA-PEI particles (Fig. 1A) were evaluated for particle size and were examined for DNA binding in vitro. Size was determined as described in Materials and Methods, showing particles generally in the range of 25–100 μm in diameter. To demonstrate DNA binding, plasmids were incubated with ethidium bromide, diluted, and added to the particles as described in Materials and Methods. Fig. 1B shows that the particles had bound the ethidium bromide-stained gel is shown.
showed <1% and ~20% positive cells in the presence and absence of serum. A panel of seven other cell lines also showed substantial levels of transfection for most of the lines using MAA-PEI under similar conditions with 10% FCS-supplemented culture medium (Fig. 4).

In vivo transfection of cells in the lung by MAA-PEI-bound plasmid

To evaluate the expression of i.v. injected MAA-PEI-bound plasmid in lung vs other tissues, a luciferase expression vector was used because of its relatively high sensitivity. After tail vein injection of 5 μg/mouse of luciferase plasmid bound to MAA-PEI, whole lung extracts showed a range of activity from 10 to 1000 pg luciferase/lung extract (extracts of nontransfected lungs had a mean background activity of 0.18 ± 0.22 pg/extract). Mice that were injected i.v. with naked luciferase plasmid alone showed only background activity in lung tissue (data not shown). Biodistribution studies in mice injected with luciferase plasmid MAA-PEI (Fig. 6A) showed that luciferase activity at 48 h was found exclusively in the lungs, with other organs having only background activities which were not distinguishable from those in control animals. Expressing the same data as ng/mg protein in the extract gave essentially the same pattern of activity distribution. Fig. 6B illustrates the time course of expression of luciferase in the lung. Activity was present by 24 h, peaked at 48 h, and continued to be detectable through 5 days but at lower levels. The transfection properties of different particle preparations showed a rough correlation between in vivo and in vitro activity.
Humoral immune responses elicited by MAA-PEI-bound plasmid

To evaluate immune responses to Ag expressed in the lung tissue, mice were injected i.v. with 5 µg of pCMV-hGH loaded on MAA-PEI particles. At biweekly intervals, sera were collected from each mouse via tail bleed. At the end of the experiment, the mice were sacrificed and BAL was performed to evaluate Ab responses in pulmonary secretions. In these experiments (Fig. 7), controls included uninjected animals and animals in which naked hGH plasmid was injected i.m. Fig. 7A illustrates the total systemic Ab response to hGH elicited by the MAA-PEI plasmid through 12 wk in one group of mice, showing a rapid and strong response from the single dose of plasmid that appeared to plateau after 4 wk. Intramuscular injection of naked DNA showed essentially the same level of Ab response systemically, and the differences from uninjected control animals for both immunized groups were statistically significant at all time points ($p < 0.02$). Fig. 7B shows a second set of mice injected with MAA-PEI-bound hGH plasmid and compared with other controls, including mice injected with the same quantity of either naked LGH plasmid or LGH plasmid in PEI-DNA complexes. MAA-PEI-hGH responses again showed high titer systemic Ab, with somewhat higher levels than were seen with naked DNA i.v., whereas the PEI-DNA complexes failed to elicit a humoral immune response.

The isotype distribution of the humoral response at 8 wk to MAA-PEI-hGH plasmid injection group is shown in Fig. 8A along with i.m. hGH and control groups, demonstrating strong IgM and total IgG response, which was dominantly IgG1 with modest IgG2a and little IgG2b or IgG3. This isotype distribution was also essentially the same at 2, 4, and 12 wk (Fig. 8B, IgG3 was not measured in the kinetic study). Because the background was slightly different for each assay and each isotype-developing reagent, the data presented are the net signal (total – the isotype-specific variation above the medium-only control at each time point), and because pooled samples were used in the kinetic study, no SDs are shown.

Mucosal Ab responses are shown in Fig. 9A, demonstrating that high titer IgA anti-hGH Ab is present in the lung secretions of only MAA-PEI-hGH plasmid-immunized mice. Fig. 9B shows the composite data from two experiments comparing particle-mediated immunization with different controls. Although a substantial systemic response was present from immunization with naked DNA by i.v. or i.m. injection (Figs. 7, B and C), very low levels of specific IgA and IgG Ab were present in either group in lung secretions, and they did not achieve statistical significance (Fig. 9B). The quantity of IgA measured in the BALF of the particle-immunized group was significantly different from the levels of IgA in other groups ($p < 0.01$) as well as from those for IgG in its own BALF and that of all other groups ($p < 0.01$). Because responses in one mucosal tissue often overlap in other mucosal tissues (45), we also examined vaginal secretions for anti-hGH IgA and IgG in the i.m. and particle-immunized female mice. As shown in Fig. 9C, low levels of IgA and IgG in vaginal secretions were detectable in particle and i.m. immunized groups, with higher IgA levels in the particle-immunized mice.

The MAA used in these experiments was prepared from HSA (due to cost of murine albumin in quantity), and anti-HSA Abs.

FIGURE 6. In vivo luciferase activity biodistribution and kinetics of expression after injection of pGL3-CMV plasmid bound to MAA-PEI. A, Biodistribution. Mice were injected via tail vein with 200 µl of PBS solution containing 5 µg of the pGL3-CMV plasmid bound to MAA-PEI particles. Data shown are compiled from 20 plasmid-injected mice and seven uninjected control mice. After 2 days, the mice were sacrificed and the lungs were isolated from all mice, and for eight mice the other organs shown were collected as well. The organs were homogenized in lysis buffer and assayed for luciferase activity. The bars represent the total nanograms of active enzyme produced per organ extract. B, Kinetics. Mice were injected as above with three mice per group and were sacrificed on different days after injection. The lungs were homogenized and assayed as in A.
were detectable, as expected, in the MAA-PEI-hGH plasmid-immunized mice (a maximum response of 1:8000 endpoint titer at 8 wk). In other experiments with animals receiving two or more administrations of MAA-PEI particles were well tolerated without anaphylactic reactions (data not shown), which is consistent with the lack of such reactions to repeated MAA injections in humans and many other animal models (other than at doses that obstruct the pulmonary circulation (46) and in a single miniature pig that developed granulomatous pneumonitis after many injections were performed over a period of 6 wk (47)).

Cytolytic immune responses elicited by MAA-PEI-bound plasmid

Elicitation of CTL was evaluated by immunizing mice with the CMV-UB#23 plasmid bound to MAA-PEI injected i.v. The plasmid encodes a protein composed of ubiquitin fused to a protein fragment containing the immunodominant epitope for gp120 in BALB/c mice (p18) (48). Eight weeks after vaccination, the spleens were harvested and assayed directly for CTL activity with various E:T ratios. As shown in Fig. 10, cytolytic T cells were present in spleen cells of the UB#23-immunized mice, and there was no nonspecific lytic activity toward the target cell without peptide loading. The lytic activity at E:T ratios of 25:1, 12.5:1, and 6.25:1 was significantly different from that of the control wells containing an E:T ratio of 25:1 but without peptide loading of the target cells (p < 0.01 for each).

Discussion

Genetic immunization has an enormous potential to broaden the scope of vaccine applications to numerous clinical diseases for which there are no currently available immunizing agents (49).

FIGURE 8. Systemic isotype immune response. A. The 8-wk pooled serum samples from the particle, i.m., and control unimmunized groups (as in Fig. 7A) were assayed to evaluate the isotype-specific Ab responses. The immunized groups had similar patterns of response, except for IgM, in which the particle group had a higher level. IgG2b, IgG3, and IgA in the immunized animals were essentially at background levels.

B. Pooled samples from the 2-, 6-, and 12-wk serum samples from the particle group were assayed to demonstrate the kinetics of the immune response with respect to isotype.
DNA vaccines have several important advantageous properties (22, 50), and if the ability to direct expression to specific tissues becomes clinically feasible, selective enhancement of localized immunity appropriate in certain diseases may also be achieved (51). The most obvious application for this approach is for infectious diseases that attack or enter through mucosal surfaces, including viral infections caused by HIV, HSV, the enteroviruses, and respiratory syncytial virus, among others. All of these viruses enter the host organism through mucosal surfaces, and vaccine immunity to them will require aggressive immune responses via effector cells in the appropriate mucosal tissues (52). In HIV, for example, even if only a few HIV particles successfully infect host target cells in the genital mucosa (where at least 75–85% of all HIV infections are transmitted (53)), the virus can rapidly reach regional lymph nodes and then widely disseminate (54).

We prepared MAA particles conjugated to a polycation, PEI, which was able to bind DNA and transfect cultured cells both in the presence and in the absence of FCS. The ability to efficiently transfect in the presence of serum permitted applications in vivo, where extracellular fluid contains serum level concentrations of albumin and other soluble proteins. We developed these particles to target the lung, because pulmonary mucosal tissues contain large numbers of interstitial APCs (macrophages and dendritic cells) (55) via which plasmid-expressed Ag could be presented to the local immune system.

In this paper, we showed that i.v. injection of plasmid-loaded particles in mice resulted in transfection of cells in the lung, whereas other tissues did not have detectable activity, in contrast to other i.v. injected preparations (56, 57). Luciferase gene expression was detected from 24 h through 5 days, with decreasing expression after 2 days, similar to what we have seen in the skin, another epithelial tissue with rapid cell turnover (F.M.O., unpublished observations), although persistence in some tissues of PCR-detectable plasmid has been demonstrated by others to last much longer (58). Ag expressed in the lung tissue would be expected to be processed subsequently by APCs to elicit immune responses through normal Ag trafficking pathways. As predicted, the expression of the plasmid in the peripheral lung tissue elicited strong immune responses. High-level systemic humoral and cytotoxic T cell responses were demonstrated. CTLs were present in the spleen, as shown by direct CTL assays, with significant activity at an E:T ratio of 6.25:1. Regarding the systemic humoral response, there was a persistent IgM response that did not decline over the 12 wk of the experiment, and there was little systemic IgA response. Such persistent IgM Ab levels have been observed in mice and...
other species (9, 59), particularly with relatively low plasmid doses and low expression of plasmids using other methods of administration (M.A.B., unpublished observations). The 5′-deoxycytidine-phosphate-guanosine (immunostimulatory) sequences present in most bacterial plasmids ordinarily bias genetic immunization responses toward the IgG2a subclass production (Th1 responses) (9, 60, 61). The small quantities of plasmid used in the MAA-PEI injection studies (5 μg) were widely distributed throughout the lung fields, thus possibly reducing the net bias toward a dominant IgG2a response via the Th1 cell activation that results from induction of local cytokine production by bacterial DNA sequences (60–62).

Indeed this relatively small quantity of plasmid (5 μg/injection) showed no inflammatory response in the lungs of injected mice, in contrast to the inflammation attributable to 5′-deoxyxytidine-phosphate-guanosine sequence effects of plasmid DNA when administered in higher doses (≥40 μg) with cationic lipids intratracheally (63). Single or multiple administrations of MAA itself at ordinary doses also does not result in detectable inflammation in humans (32) or animals (30), consistent with our observation that no inflammatory cells were observed in lung sections of MAA-PEI plasmid-injected animals.

Specific IgA Ab was found in the pulmonary BALF of immunized animals, with low level responses also detected in vaginal secretions. However, there was little systemic IgA response detected, as has been observed in some other immunization studies (64). The induction of secretory IgA in the lung is required for immunoprotection in some model systems (65), but not in others (66). Minimal mucosal responses in lung and vaginal secretions have been seen with i.m. administration of plasmid (67, 68), consistent with our data. The presence of substantial IgA in lung secretions without significant IgG also strongly suggests large local IgA production because the ratio of total IgG to IgA in lung secretions is ordinarily 2:1 or greater, with a substantial fraction of the IgG being derived from plasma (69). The presence of IgA in the vaginal secretions is expected because of the overlap among tissue sites in the common mucosal immune system (26, 70, 71). Although IgA in the vaginal secretions was likely to be locally produced, the IgG was likely to be from the circulation because as much as 50% of the IgG in cervicovaginal secretions originates from systemic sources (72).

Intravenously injected MAA-PEI particles accumulate largely in lung tissue on a first-pass basis, as shown by both the fluorescence and the expression data reported here and by the well-established clinical use of radiolabeled MAA for lung perfusion imaging in human medicine. Single administrations of MAA at ordinary doses does not result in significant inflammation in humans (32) or animals (30), although high doses that block pulmonary circulation in various animals (46) or multiple injections in miniature swine (47) have produced pathological changes in the lung. Because this agent is known to be safe and nonimmunogenic in humans through long clinical use, even with multiple doses in individual patients, intravenous injection of HSA particles could potentially be used to deliver plasmid DNA to tissues and organs other than the lung to elicit useful immune responses, e.g., in the liver (51). Selective catheterization of feeding arteries to deliver particles to tumors, for example, or to other internal organs may be useful. Other applications for gene delivery using MAA-PEI can be considered in the future, particularly as new methods for enhancing gene expression, for inducing safe gene integration into the selected target tissue cells, and for regulating the activity of desired expression vectors are developed.

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References


