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Immune responses elicited by bacterial minicells capable of simultaneous DNA and protein antigen delivery

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Abstract

Recent events surrounding emerging infectious diseases, bioterrorism and increasing multidrug antibiotic resistance in bacteria have drastically increased current needs for effective vaccines. Many years of study have shown that live, attenuated pathogens are often more effective at delivering heterologous protein or DNA to induce protective immune responses. However, these vaccine carriers have inherent safety concerns that have limited their development and their use in many patient populations. Studies using nonliving delivery mechanisms have shown that providing both protein antigen and DNA encoding the antigen to an individual induces an improved, more protective immune response but rarely, if ever, are both delivered simultaneously. Here, non-replicating bacterial minicells derived from a commensal *E. coli* strain are shown to effectively induce antigen-specific immune responses after simultaneous protein and DNA delivery. These data demonstrate the potential use of achromosomal bacterial minicells as a vaccine carrier.

Keywords: Minicells; Vaccine; Immune response; Mucosal immunity

1. Introduction

The growing concern about the realities of multidrug resistant pathogenic strains of bacteria and the alarming rate of emerging infectious diseases such as SARS (Severe Acute Respiratory Syndrome) virus, Lyme disease, and the avian flu virus strain H5N1 have stimulated the scientific community to discover novel vaccines and vaccine carriers. The recent use of *Bacillus anthracis* (anthrax) as an agent of bioterrorism in the United States has also helped in accelerating the national interest in vaccine development [1,2]. While the discovery of protective antigens remains a critical tool in vaccine development, research efforts on the design and testing of different delivery mechanisms, vaccine formulations, and overall efficacy are also needed. In studying these aspects of vaccine development, it has become increasingly clear that, while the induction of humoral immunity via parenteral administration is in some cases sufficient for protection, the best route of vaccine administration is via mucosal surfaces, especially for protection against organisms that use these surfaces as sites of entry. Efficient delivery is a concern with this approach; however, studies utilizing genetically engineered attenuated pathogenic bacteria or viruses as vaccine carriers have shown promise in a multitude of different infection and tumor models [3–5]. Although these carriers have demonstrated efficacy in animal models, administration to humans has been limited because of safety concerns ranging from the generation of adverse inflammatory responses, the threat of reversion to a pathogenic state as a result of horizontal gene transfer, and in causing cancer as has been demonstrated in the case of viral vectors [6–10].

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One potential strategy for the development of a non-living, non-attenuated vaccine carrier is the use of bacterial minicells. Bacterial minicells are small, non-dividing, achromosomal (100–400 nm) vesicles that are produced by most bacterial species as a result of premature septation. Studies focusing on the properties of *E. coli* minicells have shown that minicells can be engineered to package plasmid DNA, recombinant proteins, or both [11–13]. On a biochemical level, minicells have been shown to be metabolically active in that they can actively transport metabolites, transcribe and translate plasmid encoded gene products, and are subject to infection by bacteriophage [14–17]. In addition, minicells contain the same structural protein and lipid constituents of parental cells including lipopolysaccharide (LPS), lysophosphatidic acid (LPA), and an intact peptidoglycan layer. These surface components are quickly recognized by antigen presenting cells (APC) as pathogen associated molecular patterns through Toll-like and other receptors expressed by these cells [18–22]. The interaction between LPS and TLR4, in particular, helps to stimulate the inflammatory response necessary to generate an immune response as well as promoting the uptake and processing of bacteria [23]. Minicells retain these abilities of parental bacterial cells.

In this work, bacterial minicells derived from a commensal *E. coli* strain were used to deliver protein antigen and/or plasmid DNA to elicit antigen-specific immune responses in mice. Using green fluorescent protein (GFP) as a general antigen and monitoring antibody production in the sera as well as the mucosa, the best minicell formulation tested contained both recombinantly expressed antigen as well as plasmid DNA encoding the antigen under eukaryotic control. This delivery combination was able to induce high levels of IgG in the sera after introduction by several different routes, including intramuscular (i.m.), intranasal (i.n.), and oral (p.o.) administration. Taken together, these results suggest that with more development, minicells could be suitable as a safe and effective vaccine delivery vehicle.

2. Materials and methods

2.1. Bacterial strains and media

The MPX1B9 minicell producing *E. coli* strain [F^-, λ^-, *ilvG*, *rfb-50*, *rph-1*, *zac::aph, lacI*^{q}, P_{tac} *ftsZ20*, Δ*phoA*] was obtained from Vaxiion Therapeutics, Inc. The wild type *ftsZ* of strain MG1655 has been replaced with *ftsZ* under the control of the *tac* promoter requiring addition of 15 μM IPTG to support normal division and growth. Induction of the minicell phenotype requires that additional IPTG be added to the media as described below. MPX1B9 strains harboring pRHA-67, pRHA-67::*GFP*, pMJG3, or pGFPVAX were streaked from glycerol stock on to LB agar containing 15 μM IPTG, 50 μg/mL kanamycin and 100 μg/mL ampicillin and grown at 37 °C. Overnight cultures were grown in 3 mL of LB broth containing 15 μM IPTG, 50 μg/mL kanamycin, and 100 μg/mL ampicillin (where appropriate) while shaking at 37 °C.

E. coli Top10 cells harboring the plasmid pGFPM5 were grown on LB agar containing 100 μg/mL ampicillin at 37 °C [24]. Overnight cultures were started from isolated colonies and grown in 3 mL of LB broth containing 100 μg/mL ampicillin (LB-Amp).

2.2. Plasmid construction

The plasmids used in this study are summarized in Table 1. The rhamnose inducible bacterial expression vectors pRHA-67 and pRHA-67::*GFP* were obtained from Mpex Pharmaceuticals, Inc. and have been described elsewhere [25].

The plasmid pMJG3 was constructed by subcloning the codon optimized GFP expression cassette from pGreen Lantern into the unique *Hin*dIII site of pRHA-67. *Hin*dIII sites were introduced to this GFP cassette by PCR prior to subcloning into pRHA-67 [25]. Positive clones were identified by their ability to express GFP in cultured Cos-7 cells following Lipofectamine™ 2000 mediated transfection.

The plasmid pGFPVAX was generated by subcloning the same eukaryotic expression cassette driving GFP described above into the unique *Hin*dIII site of pRHA-67::*GFP*. Positive clones were selected by their ability to express GFP in MPX1B9 cells upon induction with rhamnose as well as the ability to express GFP in cultured Cos-7 cells following transfection with Lipofectamine™ 2000.

2.3. Minicell purification and viability

MPX1B9 strains harboring the plasmids described above were grown overnight as described and subcultured 1/250 into 400 mL of LB broth containing 15 μM IPTG, 50 μg/mL kanamycin and 100 μg/mL ampicillin. Cultures were allowed to grow to an optical density at 600 nm of 0.1 ($A_{600} = 0.1$).

Table 1
Description of plasmids used in this study

Designation	Characteristics	Source
pRHA-67	Rhamnose inducible prokaryotic expression vector, Amp^R	Mpex Pharmaceuticals, Inc.
pRHA-67::*GFP*	GFP expressed under rhamnose inducible promoter, Amp^R	Mpex Pharmaceuticals, Inc.
pMJG3	Codon optimized GFP expressed under eukaryotic (CMV) control, Amp^R	This work
pGFPVAX	GFP expressed from rhamnose inducible prokaryotic promoter and codon optimized GFP expressed under eukaryotic (CMV) promoter control	This work
pGFPM5	GFP with C-terminal his 6X tag under *tac* promoter, Amp^R	Dr. Robert Zeller [24]

MPX1B9 cells containing pRHA-67 or pMJG3 were induced to produce minicells by the addition of IPTG to a final concentration of 45 μM. MPX1B9 cells harboring pRHA-67::*GFP* and pGFPVAX were coinduced to simultaneously produce minicells and GFP protein by the addition of IPTG and L-rhamnose to final concentrations of 45 μM and 1 mM, respectively. Cultures were grown overnight for 15 h from the time of induction. Two hours before harvesting minicells, ciproflaxin was added to cultures at a final concentration of 1 μg/mL to reduce the number of viable, contaminating parent cells prior to immunization.

Minicells were harvested by differential centrifugation and linear sucrose gradients as described [26,27] using LB broth as the buffer as opposed to BSG. Quantification was carried out by reading the A_{600} of purified samples and applying the equation $A_{600} + 0.0031/5 \times 10^{-11} = \#$ of minicells/mL. Approximately 10^7 minicells were serially diluted and plated to determine purity as a function of the number of viable colonies per 10^7 minicells.

2.4. Western blots

Standard Western blotting technique was used to quantitatively determine relative amounts of GFP protein in 5×10^8 minicells. The primary antibody used was a mouse monoclonal IgG against GFP (AbCam, Cambridge, MA) diluted at 1:5000 in PBS containing 0.1% Tween 20 (PBST). Blots were incubated with primary antibody for 1 h at room temperature. Following three washes in PBST, an HRP-conjugated goat monoclonal IgG anti-mouse antibody (Sigma, St. Louis, MO) was used at a 1:10,000 dilution in PBST as the secondary antibody. Following three washes in PBST, blots were developed using the Western ECL kit (Perkin-Elmer, Boston, MA).

2.5. Cell culture and media

J774A.1 murine macrophages and RAW 264.7 murine macrophages were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) containing penicillin and streptomycin (pen/strep) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 atmosphere. J774A.1 and RAW 264.7 cell lines were obtained from the American Type Culture Collection (ATCC, Rockford, Maryland). Bone marrow derived macrophages (BMDM) and dendritic cells (BMDDC) were isolated and differentiated as described using 7 μg/mL macrophage colony stimulating factor (MCSF) or granulocyte macrophage stimulating factor (GMCSF) for the ex vivo production of macrophages and dendritic cells, respectively [28].

2.6. Minicell uptake assays

J774A.1, RAW 264.7, BMDM's and BMDDC's were seeded at a density of 10^5 in tissue culture treated four-well slide chambers (BD Biosciences, Bedford, MA) with 1 mL of described media. Cells were allowed 12 h to adhere before minicells were added. Purified minicells containing the plasmid pRHA-67 were stained for 15 min with SYBR Gold (Molecular Probes, Inc., Carlsbad, CA) at a final concentration of 1 μg/mL. SYBR Gold-stained minicells were added directly to the media and allowed to incubate for 0, 15, 30, 45, 60, and 90 min. Cells were immediately washed three times with 1 mL of PBS to remove any remaining minicells and fixed in 1 mL of 4% paraformaldehyde in PBS for 30 min at 4 °C before visualization by confocal microscopy.

2.7. Minicell mediated DNA delivery to antigen presenting cells (APC)

Purified minicells containing the plasmid pMJG3 were added to 10^5 J774A.1, RAW 264.7, BMDM or BMDDC cultured in four-well slide chambers at different multiplicities of infection (M.O.I.) and allowed to incubate for 2 h at 37 °C in 5% CO_2 atmosphere. Following incubation, minicells were removed and the cells washed three times each in sterile PBS. To ensure the killing of any remaining extracellular bacteria, cells were cultured in 750 μL RPMI-1640 media containing 4 μg/mL gentamycin and allowed to incubate at 37 °C in 5% CO_2 for 4 h. After 4 h, the media was removed and replaced by 1 mL of RPMI-1640 containing pen/strep and 10% FBS and allowed to incubate for 48 h to allow for transgene expression. After 48 h, the cells were fixed and analyzed for GFP expression by confocal microscopy.

2.8. Purification of GFP protein

E. coli TOP10 cells containing pGFPM5 were cultured as described above. The following day, a 1/250 subculture into 100 mL of LB-Amp was performed and cells were allowed to grow to mid-log phase (A_{600} 0.3–0.5) before induction of His-tagged GFP expression with 100 μM IPTG. The cultures were allowed to grow overnight while shaking at 37 °C.

His-tagged GFP was purified using the Qiagen Nickel-NTA quick elute spin kit as described by the manufacturer (Qiagen, Inc., Valencia, CA). Purified GFP was dialyzed against PBS overnight in a slide dialyzer with a 10,000 kDa molecular weight cut-off (Pierce, Rockford, IL). Protein concentration was determined by Bradford assay (Sigma) using a standard curve generated by known concentrations of BSA. Purity was determined by Coomasie staining of an SDS–PAGE gel (data not shown).

2.9. Immunizations and sample collection

Intramuscular (i.m.) immunizations: Groups of six female C57BL/six mice, 6 weeks in age, were immunized in the right quadriceps with PBS containing naked plasmid DNA (pGFPVAX), minicells containing empty vector plasmid (pRHA-67), minicells containing only soluble GFP protein (pRHA-67::*GFP*), minicells containing only a eukaryotic expression cassette driving GFP expression (pMJG3), or minicells con-

taining *both* soluble GFP protein and plasmid containing the eukaryotic GFP cassette (pGFPVAX) in a 50 μL volume of PBS. A total of 10^{10} minicells were used in each individual immunization.

Mucosal immunizations: Nasally administered (i.n.) mucosal immunizations were carried out by administration of minicells in a 20 μL volume via pipetman at 10 μL per nostril of mice anesthetized with isoflourane. For gastric (p.o.) immunizations, mice were anesthetized with isoflourane and given minicells in a 200 μL volume of sodium bicarbonate buffer (pH 9.5) with a feeding cannula. A total of 10^{10} minicells were used in each individual immunization.

Immunizations for the three dose regimen took place on days 0, 14 and 28. Mice were sacrificed on day 35. Immunizations for the single dose regimen took place on day 0 and mice were sacrificed on day 21. Mice were sacrificed by the administration of CO_2 and blood harvested by immediate cardiac puncture. To isolate sera, blood was allowed to clot for 4 h at room temperature and then at 4 °C overnight. The following day, the blood clots were removed and the resultant sera were further purified by a 1 min centrifugation at 7500 × *g* to remove any contaminating red blood cells or lymphocytes. The sera were then frozen at −80 °C for subsequent analysis.

To isolate IgA from the lungs, the tracheas of euthanized mice were exposed and the tracheal tube nicked with scissors. The lungs were washed with 1 mL of PBS containing 1 mM PMSF and 50 mM EDTA (PBS–PE) from a tuberculin syringe fitted with a 30 mm blunt end lur-lock accessory secured into the trachea by suture. The collected volume was subject to a 5 min spin at 5000 × *g* to remove any cells and cellular debris. Lung lavage fluids were stored at −80 °C prior to analysis.

To isolate IgA from the small intestine, a 15 cm section of the small intestine was removed from euthanized mice and flushed with 5 mL of PBS–PE. Samples were centrifuged for 5 min at 5000 × *g* to remove cellular debris. The supernatant was removed and IgA was precipitated and concentrated by the addition of 33% (v/v) saturated ammonium sulfate at 4 °C. The precipitate was centrifuged at 10,000 × *g* for 30 min and the supernatant discarded. The resulting pellet was resuspended in 1 mL of PBS–PE and dialyzed for 12 h. The resultant intestinal lavage was stored at −80 °C prior to analysis.

2.10. Measurement of antibody levels

The sera of immunized mice were analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of GFP specific IgG. Purified, recombinant GFP was used to coat 96-well microtiter plates at 10 μg/well overnight at 4 °C. Sera were serially diluted into the plate from 1:16 to 1:2048 and allowed to incubate for 4 h at room temperature. A 1/20,000 dilution of HRP-conjugated goat monoclonal anti-mouse IgG in PBST served as the secondary antibody. Detection was performed using the HRP substrate TMB at 100 μL/well (Pierce). Reactions were allowed to proceed until color developed in the wells and were stopped by the addition of 100 μL 1 M H_2SO_4. Plates were read at 450 nm (OD 450). Serum titers of IgG were calculated by averaging the OD 450 values at each serum dilution from individual mice (two independent experiments of $n = 3$ for a total of six mice per group). These values were then plotted against the serum dilution value from which they were obtained. Half maximal OD 450 readings were calculated by dividing the highest OD 450 in an individual group by 2. Titers are defined as the reciprocal of the average dilution factor required to achieve half maximum signal at 450 nm on the plot. The average titer value of the naïve group was subtracted as background (Fig. 2).

Lung and intestinal lavages were assayed by ELISA exactly as described for sera with the exception that the secondary antibody used was a goat anti-mouse IgA conjugated to HRP (Sigma). Results for IgA detection experiments are expressed as the average increase in absorbance readings at a 1:64 dilution of lavage fluid (Fig. 3).

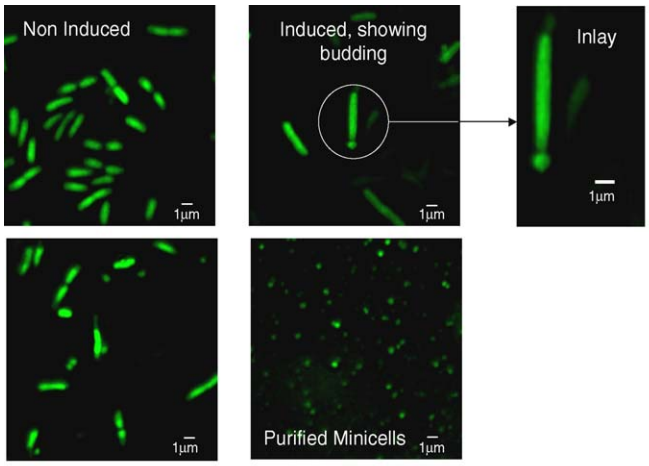
2.11. Statistical analysis

Data are presented as means ± S.E.M. Statistical significance of the differences between experimental groups was calculated by one-way ANOVA and an unpaired Student's *t*-test using GraphPad PRISM® version 4.0 software.

3. Results

3.1. Minicell vaccines can carry both heterologous proteins and eukaryotic expression plasmids

A major goal of the studies presented here is to demonstrate that the minicells produced from *E. coli* strain MPX1B9 can be utilized to produce vaccines capable of encapsulating both heterologous protein antigens as well as eukaryotic expression plasmids for delivery in vivo. To this end, MPX1B9 cells harboring the plasmid pRHA-67::*GFP* were induced to produce GFP simultaneous with minicell production. Fig. 1a shows that minicells purified as described in Section 2 were found to contain detectable levels of GFP protein using confocal microscopy. The quantification of GFP protein was performed using a series of Western blots (not shown) derived from each minicell preparation in conjunction with densitometry and the results shown in Fig. 1a. To demonstrate that minicells could also encapsulate plasmid DNA, plasmid DNA was isolated from 10^{10} minicells derived from MPX1B9 harboring either the pRHA-67, pRHA-67::*GFP*, pMJG3, or pGFPVAX plasmids. The amount of recovered plasmid DNA was quantitated using A_{260} readings. A summary of the formulation results for each plasmid is also shown in Fig. 1a. These data show that minicells can either encapsulate plasmid DNA alone or in combination with expressed recombinant protein.



Minicell formulation	GFP protein (μg)	plasmid DNA (μg)
MPX1B9 / pRHA-67	0.0 ± 0	3.4 ± 0.8
MPX1B9 / pRHA-67::*GFP*	7.6 ± 1.3	3.0 ± 0.3
MPX1B9 / pMJG3	0.0 ± 0	3.2 ± 0.7
MPX1B9 / pGFPVAX	6.8 ± 1.7	3.1 ± 0.9

(a)

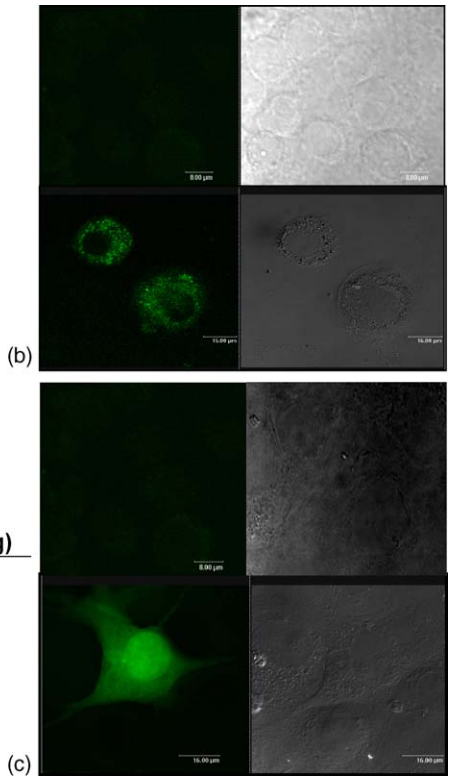


Fig. 1. Minicell production, isolation, internalization and transfer of DNA to cultured APC. (a) The minicell production process. MPX1B9 cells containing pRHA-67::*GFP* were co-induced to express soluble GFP while producing minicells. The budding and purification process was followed by visualizing GFP-containing minicells. The top left panel shows MPX1B9 cells that express GFP in the absence of inducing minicell formation. The bottom left panel shows the same culture three generations after inducing minicell production. The inlay demonstrates a budding event from the polar region of the parent cell. Purified minicells containing soluble GFP are in the bottom right panel. The table summarizes the results of minicell vaccine formulation testing. The amounts of protein antigen and plasmid DNA contained within the indicated minicell vaccines are shown. Results are from three independent experiments using 10^{10} minicells per experiment. (b) SYBR gold stained minicells were incubated with J774A.1 for various times. The lower panel shows J774A.1 cells incubated with SYBR gold stained minicells for 90 min. The addition of minicells containing no plasmid DNA was used as a negative control (top). (c) BMDDC were analyzed for GFP expression 48 h after incubating with minicells harboring the expression plasmid. The bottom panel shows BMDDC expressing the GFP transgene delivered by minicells at an M.O.I. of 10,000. APC incubated with minicells containing empty vector plasmid pRHA-67 serve as the negative control (top).

Prior to any immunization, it was important to reduce the number of viable parent cells remaining in the minicell preparation. To this end, minicell-producing cultures were treated with 1 μg/mL ciproflaxin prior to the isolation of minicells. It was determined that the number of viable contaminating parent cells was reduced from $6.0 \times 10^4/10^{10}$ minicells to $\sim$150/10^{10} minicells when cultures were treated with ciproflaxin for 2 h.

3.2. Minicell uptake by APC in culture

A major mechanism of vaccine action in eliciting a protective immune response is to deliver antigen to APC for presentation to lymphocytes. To test whether minicells have the potential to deliver heterologous protein to APC in vivo, the uptake of minicells was evaluated in several different immunologically relevant phagocytic cell lines in vitro. Minicells harboring plasmid DNA (pRHA-67) were stained with SYBR Gold and their uptake was followed at various time points. As shown in Fig. 1b, substantial numbers of minicells were taken up by J774A.1 cells at 90 min. Other cell lines tested included RAW 264.7, BMDM and BMDDC, and all exhibited the ability to phagocytose minicells with similar kinetics. As an additional control, identical experiments were performed with HeLa and Cos-7 cells. HeLa and Cos-7 cells are non-phagocytic under normal conditions. As a result, no minicell uptake was seen at any time point observed in these cell lines (data not shown). The uptake of minicells by APC in culture supports the hypothesis that minicells can deliver heterologous protein directly to APC for presentation to immune cells.

3.3. Minicells can deliver DNA to cultured APC

Once minicell uptake in cultured APC was demonstrated, we then evaluated the ability of minicells to deliver DNA for subsequent expression in vitro. Accordingly, minicells harboring the eukaryotic GFP reporter construct, pMJG3 (see Table 1), were incubated with cultured APC at various M.O.I.s for 2 h. Fig. 1c shows BMDDC expressing the GFP transgene delivered by bacterial minicells in culture. J774A.1, RAW 264.7, BMDM and BMDDC all showed sim-

ilar expression patterns (data not shown). Counterstaining APC with DAPI allowed for the quantification of the percent of cells transfected and it was found to be between 0.02 and 0.2%, depending on the cell type used. J774A.1 and RAW 264.7 cells had the highest numbers of transfectants, likely because they are mitotically active and rapidly dividing cells are more easily transfected [29]. These observations demonstrate that while transfection efficiency is not high in these cells in vitro, minicells do have the ability to deliver DNA for protein expression by APC.

3.4. Recombinant minicells elicited humoral immune responses

In cases where attenuated bacterial strains have been used as genetic vaccine carriers, transfection efficiencies in vitro do not correlate well with what will be seen in vivo, requiring in vivo studies as the true test of vaccine delivery efficacy [30]. To test for antigen-specific immune responses to GFP, mice were immunized three times each via the i.m. route with several different minicell formulations. The minicells used included those encapsulating either GFP protein alone, eukaryotic expression plasmid DNA alone, the combination of GFP protein and plasmid DNA in the same minicell, or empty vector controls. As a comparison, naked plasmid DNA (pMJG3) was administered in amounts equivalent to that encapsulated in 10^{10} minicells (~3 μg DNA) on the same immunization schedule. As seen in Fig. 2a, there was a ~12-fold increase in antibody titers from mice immunized with minicells containing both heterologous GFP protein antigen and plasmids encoding the mammalian expression cassette driving GFP expression (MC DNA/Protein). This increase was over serum titers of GFP-specific IgG from mice immunized with naked plasmid DNA alone, minicells containing GFP protein antigen alone, or minicells containing plasmid DNA vaccine alone. These results indicate that much higher antibody responses were achieved when recombinantly expressed protein antigen and eukaryotic expression plasmid DNA were delivered together by the minicell vehicle. Naïve mice and mice immunized with empty vector minicells were all negative for GFP-specific antibodies.

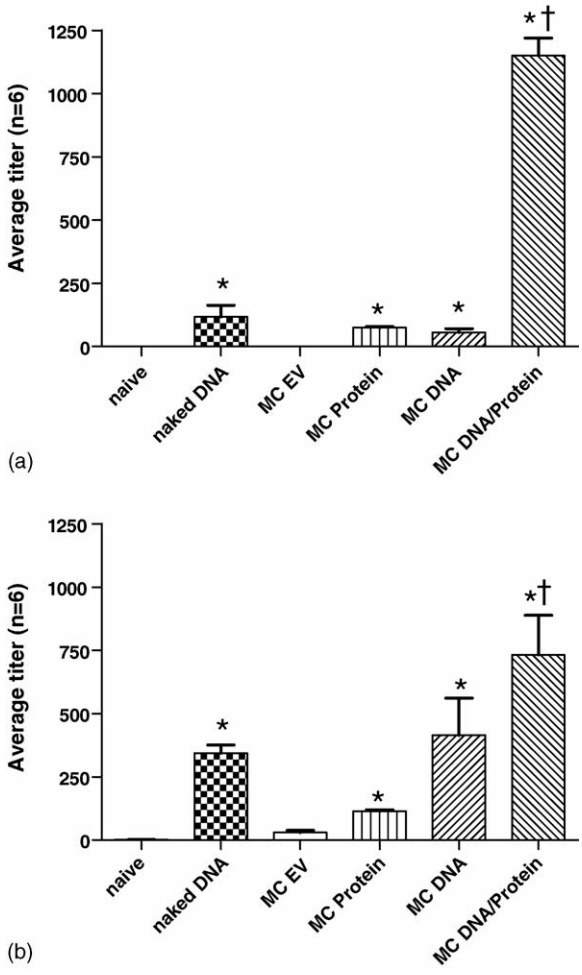


Fig. 2. GFP-specific IgG from sera of immunized mice. (a) Mice were immunized 3 times i.m. (day 0, 14, 28) with minicells containing both soluble GFP protein and plasmid DNA (MC DNA/Protein), the eukaryotic expression cassette driving GFP expression (MC DNA), recombinantly expressed GFP protein (MC Protein) or empty vector controls (MC EV). Control groups were immunized 3 times i.m. with either plasmid DNA (naked DNA) or not at all (naïve). Serum was harvested at day 35 and GFP-specific IgG antibodies were detected by ELISA. (b) GFP-specific IgG from sera is detectable from a single dose administration. Mice were immunized one time i.m. as described and GFP-specific antibody detected by ELISA on day 21. Titers for both experiments were calculated as described in Section 2. $^{*}p < 0.05$ compared against naïve group. $^{\dagger}p < 0.05$ compared against all groups tested.

3.5. Single dose i.m. immunization generates an immune response

While many vaccines require multiple doses, it is desirable to develop vaccine delivery mechanisms that require only a single administration to elicit strong immune responses. To determine if GFP-specific immune responses could also be achieved in a single dose of minicells, mice were subsequently immunized with a single i.m. administration using the same minicell formulations that were used above. Sera harvested from these mice at day 21 revealed that GFP-specific IgG antibodies were detectable following the single dose (Fig. 2b), although the titers observed were not identical to what was observed with a triple dose immunization schedule. In these experiments, DNA alone, either naked or encapsulated by minicells, was more efficient than in those shown in Fig. 2a. Nevertheless, minicells that contained both heterologous GFP protein antigen and the plasmid encoding this antigen induced a significant (2.5-fold, $p < 0.05$) increase in GFP-specific antibody over titers produced by naked plasmid DNA alone. Minicells containing protein or DNA alone were not as efficient at inducing a humoral immune response as those providing both, supporting the hypothesis that the ability of minicells to simultaneously deliver both DNA and protein may be beneficial in the development of vaccines for clinical use. These studies shown in Fig. 2 also indicate

that multiple doses delivered i.m. may result in a significant increase in titer over that observed for a single dose when minicells deliver both DNA and protein as opposed to when either alone is used.

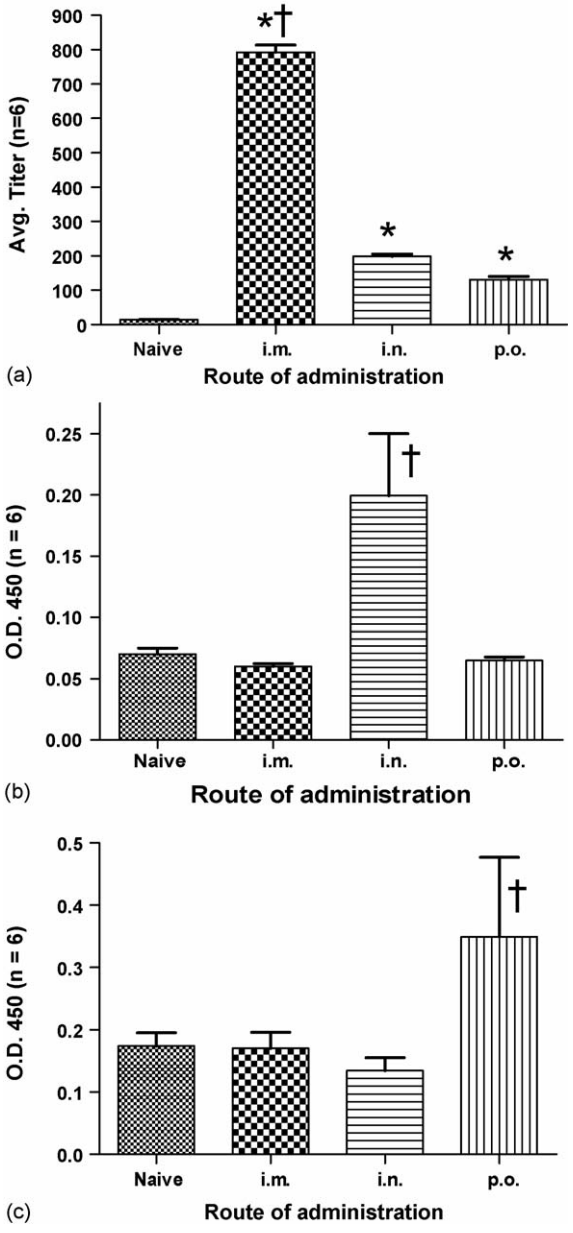


Fig. 3. GFP-specific responses from mice immunized via the intramuscular, intranasal or oral route. Mice were immunized three times (day 0, 14, and 28) each with minicells containing both soluble GFP protein and the plasmid designed for eukaryotic expression (the MC DNA/Protein vaccine of Fig. 2) by i.m., i.n. or p.o. routes. The mode of administration influences the site of Ab production. (a) GFP-specific IgG antibodies detected in the sera of immunized mice (i.m. administration demonstrates the highest titers) at day 35. (b) GFP-specific IgA antibodies detected in the nasal wash of immunized mice (i.n. administration only) at day 35. (c) GFP-specific IgA antibodies detected in the intestinal lavage fluid of immunized mice (p.o. administration only) at day 35. Serum IgG titers an relative IgA levels were calculated as described in Section 2. $^{*}p<0.05$ compared against naïve group. $^{\dagger}p<0.05$ compared against all groups tested.

3.6. Mucosal minicell immunization results in detectable immune responses

The minicell vaccine was then tested for its ability to elicit immune responses after mucosal delivery. Mice were immunized three times each with minicells containing both recombinant GFP protein and plasmid DNA encoding the eukaryotic GFP expression cassette (pGFPVAX) by i.m., i.n., or p.o. routes. These minicells were the only type tested in these experiments because of their ability to induce higher immune responses after i.m. administration (see above, Fig. 2). Results illustrated in Fig. 3a indicate that mice immunized with minicells by any of the tested routes all generated GFP-specific IgG antibody titers in serum. Intramuscular delivery showed ~4-fold increase in GFP-specific antibody titers over sera harvested from mice immunized by either the intranasal or oral routes. As expected, naïve mice showed no response. These data indicate that bacterial minicells could stimulate the formation of systemic immune responses after mucosal delivery.

Because mucosal immunizations conferring protection against pathogens typically induce the presence of antigen-specific IgA in the mucosal lining, the GFP-specific IgA content of mice immunized by the various routes was compared. Fig. 3b shows that detectable amounts of IgA were found in the lungs of mice immunized by the i.n. route, but not in the lung lavages of mice immunized p.o. or i.m. Fig. 3c illustrates the detection of GFP-specific IgA in the intestinal lavage fluid of mice immunized by the p.o. route, but not in those immunized by the other routes of administration. Although the amount produced is low, these data indicate that minicells do have the potential to induce IgA production at the expected sites of mucosal immunization.

4. Discussion

The present work demonstrates that bacterial minicells derived from the commensal *E. coli* K-12 strain, MPX1B9, can generate antigen specific immune responses in mice. Minicells capable of simultaneously delivering both the recombinantly expressed heterologous GFP protein antigen and plasmid-based GFP DNA eukaryotic expression cassette induced the greatest responses under every condition tested. Robust IgG responses were recorded following a single i.m. injection and could be increased somewhat by utilizing a three dose regimen. Few adverse effects (inflammation or infection) were observed at the site of injection in any of the mice immunized i.m. suggesting that the mice were able to tolerate the amount of LPS present on the surface of the minicells. It was also found that minicells delivering both heterologous protein and the DNA encoding the antigen under eukaryotic control could induce antigen-specific IgG antibody responses in the sera and low but detectable GFP-specific IgA in mucosal lavage fluids after a three dose regimen was administered by either the i.n. or p.o. routes.

These data demonstrate the potential of bacterial minicells as carriers for protein and/or DNA in the generation of protective, mucosally administered vaccines.

The development of non-living, non-attenuated delivery systems has major implications in vaccine development because of the potential to deliver vaccines to immune-compromised individuals, the elderly, and to children under 2 years of age [31,32]. There are several potential advantages of using minicells derived from commensal *E. coli* strains of bacteria in fitting this unmet need. Minicells derived from commensal bacteria are likely to be better tolerated than heat-killed vaccines derived from pathogenic bacteria, potentially reducing adverse inflammatory immune responses. Beyond these and other safety advantages, another potential advantage stems from the versatile nature of the minicell carrier for implementation of different "prime-boost" strategies using different vaccine modalities. The sequential delivery of genetic vaccines and protein antigens has been shown to be more effective in inducing humoral responses in humans than genetic vaccines alone [33–36] and can be well facilitated by the minicell delivery system. This strategy has proven effective using poliovirus replicons, attenuated *Salmonella enterica* sv Typhi Ty21a, and in several other genetic immunization protocols [33,34,36–38]. In all cases, the immune system was primed with the genetic vaccine and then boosted with the corresponding recombinant antigen.

Another potential advantage in using *E. coli* minicells to deliver protein antigen or DNA is that *E. coli* is a normal part of the microflora of the gut. As such, minicells derived from these strains have the ability to survive the hostile environment of the gastrointestinal tract, making them an attractive candidate for mucosal delivery. On the other hand, using minicells derived from commensal bacteria may be disadvantageous because pre-existing natural mucosal tolerance has been built to the minicell vector as a result of the exposure of the immune system to commensal bacterial antigens. As with any vaccine platform, the adjuvant properties of the vehicle may be important in generating an immune response and enhanced immunogenecity, i.e. "foreignness", may be required for efficient vaccine delivery. However, the immunogenicity of the vehicle may also prevent subsequent use of the same vehicle to deliver a different vaccine if memory responses to the vehicle have been raised in the first immunization, as has been shown in cases where *Salmonella* sp. have been used as carriers [39,40]. More testing will be required to fully evaluate the ability of commensal *E. coli* minicells to effectively deliver antigens and DNA using mucosal routes of administration. At the same time, mucosal immunity is not understood nearly as well as humoral immunity especially with respect to the development of immunological memory [41–43]. As more information becomes available on the mechanisms and requirements for the establishment of immunological memory in the mucosa, the improvements needed to enhance efficacy of minicells as vaccine carriers will be better understood.

While the data shown here directly demonstrate that minicells can induce immune responses after delivery of both heterologous protein antigen and the DNA encoding that antigen, many questions remain. For example, the studies shown here strongly suggest that multiple immunizations are superior when minicells deliver both DNA and protein. This may be because the DNA component primes the animal to respond to subsequent exposure to protein and vice versa. On the other hand, a single dose administration of naked DNA alone or of minicells containing only plasmid DNA gave higher specific antibody titers compared to those of mice immunized three times (see Fig. 2b). It is possible that this is due to the timing of the testing (21 days for single administration versus 35 days for 3 immunizations), but further experimentation is required to determine if this is the case. Either way, these results suggest that a boost is not necessarily effective in increasing antigen specific serum IgG levels when naked DNA or minicells encapsulating DNA are used. This may be due to the effects of vector priming as has been shown for certain *Salmonella* serovars used to deliver vaccines [39]. In those studies, it was shown that a previous exposure to the relevant *Salmonella* carrier resulted in dramatic decreases in immune responses to antigens delivered at a later time by the same carrier. The results shown in Fig. 2 also suggest that using minicells to deliver only soluble protein antigens is largely ineffective. This may be because soluble GFP protein is only mildly immunogenic and is located on the interior of minicells. Exchanging GFP for a more immunogenic cytosolic antigen like LT-B or expressing protein antigens on the surface of minicells may help in increasing antibody titers [44]. Future studies utilizing a more biologically relevant antigen are required to elucidate the mechanisms behind these results in addition to determining the ability to protect as well as the longevity and isotype profiles of any antibody responses.

Given that the minicell system can be engineered to produce antigen-specific immune responses, the next logical step is to demonstrate the generation of protective immunity by performing live pathogen challenge experiments in mice. Ultimately, the goal is to develop minicell vaccines that provide protective immunity after mucosal immunization against pathogens of both bacterial and viral origin.

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References

[1] Dennis C. The bugs of war. Nature 2001;411:232–5.

[2] Stephenson J. Experts focus on infective agents of bioterrorism. Jama 2002;287:575–6.
[3] Djavani M, Yin C, Xia L, Lukashevich IS, Pauza CD, Salvato MS. Murine immune responses to mucosally delivered *Salmonella* expressing Lassa fever virus nucleoprotein. Vaccine 2000;18:1543–54.
[4] Brown WR. Enteric immunization: promises and challenges. Digest Dis 1996;14:192–200.
[5] Fooks AR. Development of oral vaccines for human use. Curr Opin Mol Ther 2000;2:80–6.
[6] Davison J. Genetic exchange between bacteria in the environment. Plasmid 1999;42:73–91.
[7] Davison J. Towards safer vectors for the field release of recombinant bacteria. Environ Biosafety Res 2002;1:9–18.
[8] Dzidic S, Bedekovic V. Horizontal gene transfer-emerging multidrug resistance in hospital bacteria. Acta Pharmacol Sin 2003;24:519–26.
[9] Donsante A, Vogler C, Muzyczka N, Crawford JM, Barker J, Flotte T, et al. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. Gene Ther 2001;8:1343–6.
[10] Holmgren J, Czerkinsky C, Eriksson K, Mharandi A. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine 2003;21(Suppl 2):S89–95.
[11] Khachatourians GG, Berezowsky. Expression of recombinant DNA functional products in *Escherichia coli* anucleate minicells. Biotech Adv 1986;4:75–93.
[12] Clark JE, Curtiss RC. Analysis of recombinant DNA using *Escherichia coli* minicells. Methods Enzymol 1983;101:347–62.
[13] Rashtchian A, Brown SW, Reichler J, Levy SB. Plasmid segregation into minicells is associated with membrane attachment and independent of plasmid replication. J Bacteriol 1986;165:82–7.
[14] Crooks JH, Ullman M, Zoller M, Levy SB. Transcription of plasma DNA in *Escherichia coli* minicells. Plasmid 1983;10:66–72.
[15] Hollenberg CP, Kustermann-Kuhn B, Royer HD. Synthesis of high molecular weight polypeptides in *Escherichia coli* minicells directed by cloned *Saccharomyces cerevisiae* 2-micron DNA. Gene 1976;1:33–47.
[16] Ponta H, Reeve JN, Pfennig-Yeh M, Hirsch-Kauffmann M, Schweiger M, Herlich P. Productive T7 infection of *Escherichia coli* F+ cells and anucleate minicells. Nature 1977;269:440–2.
[17] Shepherd N, Dennis P, Bremer H. Cytoplasmic RNA polymerase in *Escherichia coli*. J Bacteriol 2001;183:2527–34.
[18] Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. C R Biol 2004;327:581–9.
[19] Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335–76.
[20] Takeda K. Toll-like receptor. Nihon Rinsho Meneki Gakkai Kaishi 2005;28:309–17.
[21] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1–14.
[22] van Duin D, Medzhitov R, Shaw AC. Triggering TLR signaling in vaccination. Trends Immunol 2005.
[23] Otterbein LE, May A, Chin BY. Carbon monoxide increases macrophage bacterial clearance through Toll-like receptor (TLR)4 expression. Cell Mol Biol (Noisy-le-grand) 2005;51:433–40.
[24] Zeller RW, Weldon DS, Pellatiro MA, Cone AC. Optimized green fluorescent protein variants provide improved single cell resolution of transgene expression in ascidian embryos. Dev Dyn 2005;235(2):456–67.
[25] Giacalone MJ, Gentile AM, Lovitt BT, Berkley NL, Gunderson CW, Surber MW. Toxic protein expression in *E. coli* using a new tightly regulated and tunable promoter system. Biotechniques 2006;40:355–64.
[26] Frazer AC, Curtiss 3rd R. Production, properties and utility of bacterial minicells. Curr Top Microbiol Immunol 1975;69:1–84.
[27] Frazer AC, Curtiss 3rd R. Derepression of anthranilate synthase in purified minicells of *Escherichia coli* containing the Col-trp plasmid. J Bacteriol 1973;115:615–22.
[28] Bastos KR, de Deus Vieira de Moraes L, Zago CA, Marinho CR, Russo M, Alvarez JM, et al. Analysis of the activation profile of dendritic cells derived from the bone marrow of interleukin-12/interleukin-23-deficient mice. Immunology 2005;114:499–506.
[29] Grillot-Courvalin C. Wild-type intracellular bacteria deliver DNA into mammalian cells. Cell Microbiol 2002;4:177–86.
[30] Fennelly GJ, Khan SA, Abadi MA, Wild TF, Bloom BR. Mucosal DNA vaccine immunization against measles with a highly attenuated *Shigella flexneri* vector. J Immunol 1999;162:1603–10.
[31] Molina V, Shoenfeld Y. Infection, vaccines and other environmental triggers of autoimmunity. Autoimmunity 2005;38:235–45.
[32] Schattner A. Consequence or coincidence? The occurrence, pathogenesis and significance of autoimmune manifestations after viral vaccines. Vaccine 2005;23:3876–86.
[33] Doria-Rose NA, Haigwood NL. DNA vaccine strategies: candidates for immune modulation and immunization regimens. Methods 2003;31:207–16.
[34] Eo SK, Gierynska M, Kamar AA, Rouse BT. Prime-boost immunization with DNA vaccine: mucosal route of administration changes the rules. J Immunol 2001;166:5473–9.
[35] Moingeon P. Strategies for designing vaccines eliciting Th1 responses in humans. J Biotechnol 2002;98:189–98.
[36] Ramshaw IA, Ramsay AJ. The prime-boost strategy: exciting prospects for improved vaccination. Immunol Today 2000;21:163–5.
[37] Viret JF, Favre D, Wegmuller B, Herzog C, Que JU, Cryz Jr SJ, et al. Mucosal and systemic immune responses in humans after primary and booster immunizations with orally administered invasive and noninvasive live attenuated bacteria. Infect Immun 1999;67:3680–5.
[38] Novak MJ, Moldoveanu Z, Huang WQ, Jackson CA, Palmer MT, McPherson SA, et al. Intramuscular immunization with poliovirus replicons primes for a humoral and cellular immune response to soluble antigen. Viral Immunol 2003;16:169–82.
[39] Vindurampulle CJ, Attridge SR. Impact of vector priming on the immunogenicity of recombinant *Salmonella* vaccines. Infect Immun 2003;71:287–97.
[40] Metzger WG, Mansouri E, Kronawitter M, Diescher S, Soerensen M, Hurwitz R, et al. Impact of vector-priming on the immunogenicity of a live recombinant *Salmonella enterica* serovar typhi Ty21a vaccine expressing urease A and B from *Helicobacter pylori* in human volunteers. Vaccine 2004;22:2273–7.
[41] Cheroutre H. Starting at the beginning: new perspectives on the biology of mucosal T cells. Annu Rev Immunol 2004;22:217–46.
[42] Cheroutre H, Madakamutil L. Acquired and natural memory T cells join forces at the mucosal front line. Nat Rev Immunol 2004;4:290–300.
[43] Cheroutre H, Madakamutil L. Mucosal effector memory T cells: the other side of the coin. Cell Mol Life Sci 2005;62:2853–66.
[44] Takahashi I, Marinaro M, Kiyono H, Jackson RJ, Nakagawa I, Fujihashi K, et al. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. J Infect Dis 1996;173:627–35.