Polylactide-co-glycolide (PLG) microparticles modify the immune response to DNA vaccination

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Keywords: Vaccination, viral, eosinophils, antigen presentation/processing, lung

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Abbreviations: PLG; polylactide-co-glycolide

Summary

Priming with the major surface glycoprotein G of respiratory syncytial virus (RSV) expressed by recombinant vaccinia leads to strong Th2 responses and lung eosinophilia during viral challenge. We now show that DNA vaccination in BALB/c mice with plasmids encoding G attenuated RSV replication but also enhanced disease with lung eosinophilia and increased IL-4/5 production. However, formulating the DNA with PLG microparticles reduced the severity of disease during RSV challenge without significantly lessening protection against viral replication. PLG formulation greatly reduced lung eosinophilia and prevented the induction of IL-4 and IL-5 during challenge, accompanied by a less marked $CD4^+$ T cell response and a restoration of the $CD8^+$ T cell recruitment seen during infection of non-vaccinated animals. After RSV challenge, lung eosinophilia was enhanced and prolonged in mice vaccinated with DNA encoding a secreted form of G; this effect was virtually prevented by PLG formulation. Therefore, PLG microparticulate formulation modifies the pattern of immune responses induced by DNA vaccination boosts $CD8^+$ T cell priming and attenuates Th2 responses. We speculate that PLG microparticles affect antigen uptake and intracellular routing, thereby influencing the outcome of DNA vaccination.

Introduction

The introduction of novel and effective vaccines has contributed substantially to reducing or eliminating the burden of infectious diseases in human and animal populations. However, it has become clear that long-established pragmatic methods of vaccine development are failing to deliver vaccines against many important diseases and that new, immunologically informed, approaches need to be taken. A particularly important current trend is the introduction of new and specific adjuvants that not only augment protective immune responses, but also modify antigenic profiles to induce less pathogenic responses[1].

The recent success and licensing of vaccines containing virus-like particles (e.g. papilloma virus antigens; Gardasil, Merck & Co., Inc.) highlights the potential of microparticulate formulations to improve immunogenicity. It has been shown that synthetic cationic microparticles comprising cationic (polylactide-co-glycolide; PLG) microparticles have strong adjuvant effects and immune-stimulating properties[2] and to induce Th1- biased immune responses[3]. PLG can adjuvant the effects of DNA and ssRNA vaccination both in the mouse and in the monkey models[4],[5] and against viral antigens [6;7]. In studies of DNA vaccination against human immunodeficiency virus (HIV) *gag* and *env* in rhesus macaques, PLG formulation increased CD8+ T cell responses up to 1000 fold while boosting total antibody responses. The magnitude of the response to DNA vaccination with PLG DNA microparticles was found to be equivalent to that induced by the highly immunogenic recombinant gp120 protein formulated with the strong adjuvant

MF-59 [8]. However, the ability of PLG-DNA formulations to skew immune responses away from Th2 is less established and has not been tested in animal models of viral pulmonary infection.

Respiratory syncytial virus (RSV) is an important common cold virus of man, and causes most cases of infantile bronchiolitis. Bronchiolitis is the commonest cause of infant hospitalisation in the western world, leading to admission of 1-2% of all children during the first year of life, and is associated with airway hyperreactivity, recurrent wheeze and the diagnosis of asthma during later childhood [9]. Asthma is characterised by a bias towards Th2 responses, associated frequently with airway eosinophilia. In a mouse model of RSV disease, sensitisation with recombinant vaccinia expressing the viral glycoprotein G primes for a strong Th2 biased immune response that causes lung eosinophilia during intranasal infection with RSV[10]. G can be mutated at the second initiation codon to prevent secretion of the glycoprotein, and sensitisation with recombinant RSV expressing only the membrane bound (non-secreted) form of the G protein induces significantly less of an eosinophilic response during RSV challenge[11]. Similarly, challenge with genetically engineered RSV lacking the ability to produce secreted G causes less eosinophilia [11;12]. It has been shown that DNA-G immunization is able to produce significant neutralising and balanced antibody responses inducing both IgG1 and IgG2a subtypes, as well as protect against RSV infection in BALB/c mice [13;14], It can reduce mucin gene expression and lessen airway hyperresponsiveness [15]. Studies of DNA vaccination against RSV infection

have therefore shown promise, but have yet to translate into safe, immunogenic and effective vaccines for use in infants.

Since DNA vaccine immunogenicity can be significantly improved by formulation with PLG microparticles in other situations, we formulated plasmids expressing various RSV proteins with PLG and tested for immunogenicity and pathogenicity in BALB/c mice. The plasmid encoding the viral glycoprotein G induced strong immune responses that were partially protective against viral challenge. However, vaccination with untreated DNA encoding G caused high levels of IL-5, IL-4 and eosinophil production during challenge; these effects were attenuated by formulation with PLG without significantly affecting antibody responses. Further, DNA encoding secreted G was even more prone to induce lung eosinophilia during challenge, an effect virtually abolished by DNA adsorption onto PLG. Therefore, DNA formulation with PLG microparticles modifies the host immune response, attenuating the pathogenic effects and modifying the host immune responses away from Th2 and lung eosinophilia without affecting the protection against viral replication.

Results

DNA vaccination and RSV challenge

Groups of 5 mice were immunized i.m. in the hind leg with 50µg of native DNA-G, or 50µg of DNA-G adsorbed onto PLG microparticles, boosted similarly after 4 weeks. Four weeks after boosting, mice were challenged by RSV infection i.n. Control groups were non-immunized, or had previously been infected with RSV i.n. (i.e. to produce protection against RSV challenge). In some experiments, control animals were immunized with empty PLG microparticles or with PLG coated with irrelevant DNA.

Mice undergoing i.n. re-infection showed a transient weight loss on days 2 and 3, consistent with passive reaction to viral antigen exposure (Fig. 1A). However, such mice showed no evidence of viral replication on day 4 (Fig. 1B), consistent with protection against viral replication. Mice undergoing primary RSV infection showed no weight loss during the first 4 days (Fig. 1A), but developed a high viral load in the lungs (Fig. 1B). By day 7, these mice had lost 22% of the starting body weight (Fig. 1A), attributable to virus-induced lung pathology [10]

Vaccination with DNA-G accelerated weight loss during the early stages following viral challenge but the maximum weight loss (day 7) was similar to mice undergoing primary RSV infection. The rate of onset and peak of weight loss was reduced by formulation of DNA with PLG (Fig. 1A), and PLG did not

significantly lessen the protective effect of vaccination on viral load during challenge (Fig. 1B).

Immune responses to infection

Although PLG formulation with DNA reduced the severity of weight loss on day 4, the enhancement of lung cellularity at this time point was unaffected (Fig. 2A). However, lung eosinophilia was reduced by approximately 50% by addition of PLG (Fig. 2B), with virtual elimination of the IL-5 and IL-4 detected in the supernatant of isolated lung cells (Fig. 2D, 2F). There was also a similar effect on TNF-α levels detected (Fig. 2E). Despite this clear effect on IL-4 and IL-5, PLG had no effect on production of IFN-γ (Fig. 2C). Vaccination with DNA-G, with or without formulation with PLG, induced a significant RSV specific antibody response in the serum (Fig. 3A). Levels of total antibody and IgG isotypes were broadly similar after vaccination with DNA-G, regardless of the presence of PLG (Fig. 3B, C). Additionally, neutralizing ability of serum from mice immunized with either construct was similar (data not shown). In experiments where empty PLG or PLG/irrelevant DNA were used, immune responses mirrored those in primary RSV infection (data not shown).

Analysis of the phenotype of cells recovered from the lungs of mice on day 8 after viral challenge showed that mice undergoing primary infection had responses dominated by $CD8^+$ T cells, which outnumbered $CD4^+$ T cells in ratio of approximately 1.5:1 (Fig. 4B). Vaccination with DNA-G boosted $CD4^+$ T cell

responses and reduced CD8+ T cell responses, reversing this ratio. However, formulation of the DNA with PLG caused more balanced responses with $CD8^{+}$:$CD4^{+}$ T ratio 1.3:1 (Fig. 4).

Immune responses to secreted antigen

The amino acid sequence of the RSV G protein can be manipulated to induce expression of a truncated protein that is purely secreted (Gsec), mimicking a form of the RSV G protein naturally produced early after RSV infection. To test the effects of DNA-Gsec in this system, mice were similarly vaccinated with constructs with or without formulation to PLG.

Weight loss following RSV challenge mirrored that observed after vaccination with the standard plasmid encoding wild type G, and formulation to PLG reduced the severity of disease during RSV challenge. However, PLG formulation had no effect on the peak of weight loss or cellularity in the BAL. Vaccination with DNA-Gsec caused marked lung eosinophilia on day 4 (Fig. 5D), and this eosinophilia persisted and actually increased on day 8 (Fig. 5E), although RSV has been cleared from the lung by this time point (data not shown). Remarkably, PLG formulation of DNA-Gsec greatly reduced eosinophilia on day 4 (Fig. 5D) and abolished the persistent and enhanced eosinophilia on day 8 (Fig. 5E). This was associated with a significant enhancement of CD8 T cell responses on day 8 (Fig. 5G).

Cytokine analysis revealed exactly the same pattern as in experiments where wtG was used for DNA and DNA/PLG formulations (data not shown).

Discussion

Although DNA vaccination has been successful in some experimental settings, improved formulations and immunogenicity are required before it can be successfully applied in man. We show here that plasmid DNA encoding the RSV glycoprotein G is immunogenic in mice, but that it also produces a Th2 biased response with lung eosinophilia during challenge. This effect is particularly marked with plasmid encoding the secreted form of G. In either case, formulation of DNA with PLG microparticles greatly attenuated the pathogenic effects of DNA vaccination without significantly lessening partial antiviral protection. This represents a significant advance, suggesting that microparticulate formulation can be used to manipulate host immune responses to DNA vaccination.

The induction of significant eosinophilia during RSV challenge of DNA vaccinated mice is consistent with other studies demonstrating that the G protein can sensitise for eosinophilia [10;11;16]. However, there are conflicting results from studies using DNA plasmids encoding G as a vaccine. Balanced Th1/Th2 responses accompanied by significant protection against RSV challenge and an absence of pulmonary eosinophilia have been reported in some settings [13;14]. However, sensitisation with DNA encoding G or F are both reported to induce BAL eosinophilia [17]. Differences in DNA expression vectors, route of immunization, dose and vaccination schedule may influence these different outcomes.

The mouse model of RSV is ideal for studies of this type, in that the immune response can be selectively biased towards Th1 or Th2 immunity, to favour cytotoxic T cell responses or antibody production. In this case, we chose to study immune responses that favour Th2 cytokine production and lung eosinophilia, allowing us to study the effects that counter this pattern of immunity. We reasoned that DNA formulation with PLG microparticles might lead to phagocytosis by antigen presenting cells, and that the intrinsic adjuvant properties of DNA (due to un-methylated CpG motifs) might significantly alter the immune response in ways that would inhibit pathogenic effects of vaccination. The presence of CpG motifs is associated with an increase in IL-12 mRNA expression resulting in a Th1-biased microenvironment [18]. CpG motifs present in bacterial DNA specifically bind Toll-like receptor-9 (TLR-9) located on the internal membranes of several cell types including B cells, macrophages and dendritic cells. Such interactions activate the Toll/interleukin (IL)-1-recptor signalling pathway leading to production of pro-inflammatory cytokines including the Th1-polarising cytokine, IL-12. Furthermore, physical trauma due to the skin following needle injection leads to an increase in TLR-9 mRNA expression [19], enhancing the response to CpG motifs [20]. Therefore, immunization with PLG microparticles that delivers DNA directly to endosomes would favour enhanced Th1 responses, counteracting the intrinsic Th2 bias induced by immunization with RSV glycoprotein G. We and others have shown that $CD8^+$ cells producing IFN-γ attenuate eosinophilia by inhibiting Th2 responses [21;22]. This effect may result

from PLG facilitating entry of material into the cytosol, favouring presentation via the endogenous class I pathway [23;24].

Our results show that formulation with PLG boosts $CD8^+$ responses in the lung during subsequent RSV infection, suggesting that PLG deviates responses towards MHC class I antigen 'endogenous' presentation to CD8 T cells. We suggest that PLG may promote direct uptake of DNA into APCs, resulting in endogenously expressed protein that is processed and presented by the MHC class I pathway for the induction of CD8+ T cells [8;25;26].

Intramuscular injection of DNA primarily targets myocytes, where antigen is expressed before release and uptake by APCs. APCs may also be directly transfected, but this is thought to be a less important mode of action. PLG forms a depot at the site of injection lasting for at least 7 days, accompanied by expression of encoded antigen [27]. The mechanical trauma of injection and presence of PLG recruits phagocytes and activates antigen presenting cells at the injection site. We speculate that PLG may not only affect the DNA which is co-administered, but that residual PLG could also influence the uptake of antigen synthesised by non-professional antigen presenting cells. However, our data does not allow us to differentiate between these possibilities. Although our findings demonstrate the ability of PLG formulation to modify responses to DNA vaccination, we emphasise that this type of vaccine is unlikely to be effective in children in preventing RSV disease, and that other types of vaccine (live attenuated or

mucosal vaccines, for example) may offer greater practical hope in preventing RSV infection in man.

To conclude, DNA vaccines formulated with PLG avoid the enhanced disease seen after simple DNA vaccination by modulating pathogenic Th2 responses and by enhancing Th1 effects. This may represent an enhancement of the adjuvant activity of CpG motifs naturally contained within DNA, or be caused by effects on antigen processing and presentation. PLG formulation could therefore improve the outcome of vaccination, leading to greater vaccine efficacy and safety. These effects might be particularly beneficial in neonatal vaccination (a time at which RSV vaccines should ideally be given), since newborn children show an inherent deficiency of Th1 responses to antigenic challenge.

Materials and Methods

DNA plasmids

The wild type sequence of the G protein of RSV strain A2, was cloned into the *Sal*I and *Xba*I restriction sites of the eukaryotic expression vector pCMVKm2 that contains the cytomegalovirus (CMV) immediate-early enhancer/promoter and bovine growth hormone (bGH) terminator (Chiron Corporation, Emeryville) [28], resulting in the plasmid pCMVKm2_wtG; from hereon in referred to as DNA-G. The plasmid was grown in *Escherichia coli* strain XL-1 blue (Stratagene), purified using a Qiagen Endofree Plasmid Giga kit (Qiagen) and resuspended in nuclease free water (Promega).

Preparation and formulation with PLG microparticles

Cationic microparticles of about 1µm diameter were prepared using a modified solvent evaporation procedure, as described [29]. A primary emulsion of PLG polymer (RG504) was homogenized at 6% w/w with dichloromethance (DCM) in the presence of tris EDTA using an IKA homogeniser. This preparation was then added to a secondary emulsion of purified water containing 0.015% cetyltrimethylammonium bromide (CTAB) resulting in 1% CTAB w/w PLG. The resulting water-in-oil-in-water emulsion was then stirred overnight to allow evaporation of DCM. Microparticles were then filtered and DNA adsorbed by adding a 2mg/ml solution drop wise at approximately 100µl/min. Formulations were lyophilised for storage prior to use. Physical characteristics were examined as previously described [29].

Immunization of mice

Groups of five 6 to 8 week old female BALB/c mice (Harlan, Berkhamsted, UK) were housed under pathogen-free conditions. All procedures were subjected to ethical review and licensed by the Home Office, UK. A total of 50µg of either PLG/DNA-G formulation or naked DNA-G was administered in a total of 100µl volume between each hind leg by needle injection. All mice were boosted with the same dose of vaccine after 4 weeks. A further 4 weeks after the second dose, all mice were anaesthetized and given 100µl $5x10^5$ pfu RSV (strain A2) via the nose. The virus was grown and assayed for infectivity on Hep-2 cells [21]. All stocks were free of mycoplasma contamination.

Quantification of viral RNA

RNA was extracted from the lung using RNA STAT-60 (Tel-test Inc.) and cDNA was generated with random hexamers using an Omniscript RT-kit (Qiagen). PCR specific for RSV L gene was performed at 50°C for 2 min and 95°C for 10 min followed by 40 two-step cycles (95°C for 15s, 60°C for 1min) with Quantitech Probe PCR kit (Qiagen) and 900 nM forward primer (5'-GAACTCAGTGTAGGTAGAATGTTTGCA – 3'), 900 nM reverse primer (5' – TTCAGCTATCATTTTCTCTGCCAAT – 3') and 100 nM probe (5' – FAM – TTTGAACCTGTCTGAACATTCCCGGTT – TAMRA – 3'). Copy number of the L gene was determined from standard curves against PCDNA3 plasmid vector containing a fragment of the RSV L-gene. PCR amplifications were measured in

real time using ABI 7000 (Applied Biosystems) and analyzed using Sequence Detections Systems (v1.1).

RSV-specific antibody ELISA

ELISA antigen was prepared by infecting HEp-2 cells with RSV strain A2 at 1 PFU/cell. When a significant cytopathic effect was observed, infected cells were harvested, centrifuged at 400×g, resuspended in 3 ml distilled water and then subjected to 2 min of sonication (Ultrawave Ltd., Cardiff, GB); 50 µl aliquots were stored at -20°C until required. Microtiter plates were coated overnight with 100 µl of a 1:500 dilution of either RSV antigen or HEp-2 cells alone. After blocking with PBS/1% BSA for 1 h, dilutions of test samples (diluted in PBS containing 0.2% BSA 0.01% Tween) were added for a further 2 h at 37°C. Bound antibody was detected using peroxidase-conjugated rabbit anti-mouse Ig and *O*-phenylenediamine as a substrate (Sigma Fast OPD tablets). For isotype specific antibody determination, bound antibody was detected with either biotinylated anti-IgG1 or IgG2a as required, followed by extravidin peroxidase (Sigma) before colour development with OPD. Colour development was stopped with 2 M H_2SO_4, and optical densities were read at 490 nm. The level of RSV-specific antibody was determined by subtracting the absorbance obtained from samples incubated on RSV antigen-coated wells from the same sample incubated on HEp-2-coated wells.

ELISA for interleukins

IL-4, IL-5, TNFα and IFNγ were measured in supernatants of homogenized lung cells by ELISA performed according to the manufacturer's instructions (Becton Dickinson-Pharmingen). Briefly, ELISA plates (Nunc) were coated with a capture antibody diluted 1:500 and left overnight at 4°C. The wells were then washed with PBS–0.05% Tween 20 and blocked with PBS–1% BSA for 2 h at room temperature. Samples or standard were added at 100μl per well and left overnight at 4°C. Bound cytokine was detected with a biotinylated anti-cytokine antibody, Avidin-horseradish peroxidase, and OPD. Colour development was stopped with 2 N H_2SO_4, and optical density read at 490 nm. The concentration of cytokine in each sample was determined from a standard curve.

Cell surface markers

Lung cells were plated at 10^6 cells/ml in FACS staining buffer (PBS 1%BSA 0.1% Azide). Cells were then blocked with Fc block (CD16/CD32 antibody from Pharmingen) prior to staining with fluorescently labelled antibody against surface markers (CD3-FITC, CD-APC, CD8-PECy5, all from Pharmingen) for 30 min on ice and then fixed for 20 min at room temperature with 2% formaldehyde. Cells were then analyzed on a BD-LSR flow cytometer collecting data on at least 50,000 events.

Statistical analysis

Differences between groups were analysed using one-way ANOVA followed by the Tukey test using GraphPad Prism (Version 4.0). For multiple comparisons

between groups, two-way ANOVA was performed followed by the Bonferroni post test. P values <0.05 were considered statistically significant.

Acknowledgements:

Supported by the Medical Research Council (UK) CASE awards G78/8103 and G78/5996; EU grant ‘Impressuvac’ (QLRT-PL1999–01044) and Wellcome Trust Programme Grant 071381/Z/03/Z.

Figure legends

Figure 1: Effects of immunization on protection from RSV-induced weight loss. Mice were immunized twice i.m. with either PLG/DNA-G (black circles) or DNA-G (white circles) 4 weeks apart; control mice received either nothing (1° RSV: black squares) or i.n. RSV once (2° RSV: black triangles). 4 weeks after the second dose, all mice were challenged i.n. with RSV. A) Change in body weight post infection. B) RSV L gene copy number per lung as measured by Taqman RT-PCR. Data points represent the mean of n=5 ± SEM: BLD, below limit of detection; N/S, not significant.

Figure 2: PLG/DNA-G induces a weaker Th2 response than naked DNA-G. Immunized mice were challenged with RSV. Four days later, mice were sacrificed and BAL performed. The total viable cell number was calculated by trypan blue exclusion (A) and the percentage of eosinophils calculated from H&E stained cytospin slides (B). Lungs were removed, mashed and supernatants processed for cytokine ELISA to detect IFN-γ (C), IL-5 (D), TNF-α (E) and IL-4 (F). Data shown as mean +/- SEM.

Figure 3: Effect of immunization on the humoral response to infection. Eight days after RSV challenge, mice were sacrificed and serum tested for viral antibody. Total RSV-specific IgG (A), IgG1 (B) and IgG2a (C) isotypes were

measured by ELISA. Data show the average and standard error for optical densities obtained after development with OPD for serum diluted 1 in 400.

Figure 4: PLG/DNA-G induces a stronger CD8 response than naked DNA-G. Eight days after RSV challenge, mice were sacrificed and lungs taken for FACS analysis. Cells were stained with fluorochrome-conjugated antibodies to CD4, CD8 and CD3. Positive events were recorded on a BD-LSR and cells gated on lymphocytes based on forward and side scatter characteristics. Individual representative dot plots are shown for each group (A) from which the group mean and standard error was determined (B).

Figure 5: **Effects of PLG formulation on immune responses to DNA encoding secreted RSV G protein.** Mice were immunized twice i.m. with either PLG/DNA-Gsec or DNA-Gsec 4 weeks apart; control mice received either nothing (1° RSV) or i.n. RSV once (2° RSV). Four weeks after the second dose all mice were challenged i.n. with RSV. Four and eight days after RSV challenge mice were culled and BAL and lungs collected. (A) Change in body weight post infection. Mice receiving PLG/DNA-Gsec (black diamonds) or DNA-G sec (white squares), 1° RSV (black circles) and 2° RSV(black squares) (B) Cell counts in BAL, 4 and (C) 8 days after challenge, (D) eosinophilia in BAL 4 and (E) 8 days after challenge, (F) CD8 T cells in lung 4 and (G) 8 days after challenge. Bars represent mean of n=5 mice ± SEM.

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