Immunity against both polyomavirus VP1 and a transgene product induced following intranasal delivery of VP1 pseudocapsid–DNA complexes

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Murine polyomavirus VP1 virus-like particles (VLPs) can bind plasmid DNA and transport it into cells both in vitro and in vivo. Long-term expression of the transgene can be observed, suggesting that VP1 VLPs may be used as DNA delivery vehicles for gene therapy. In this study we have analysed the in vitro efficiency of transfection using different DNA/VLP molar ratios and the immune response induced following intranasal administration of these complexes to mice. Our results indicate that in short-term in vitro culture VP1 VLP–DNA complexes appear to be as efficient as DNA alone at transfecting cell monolayers. They also show that VP1 VLPs are very immunogenic, inducing high proliferative cell responses and both serum and mucosal antibodies. Moreover, VP1 VLP–DNA complexes appear to be capable of inducing a stronger immune response to the transgene product (β-galactosidase) than immunization with DNA only. The results suggest that polyomavirus VP1 VLPs derived from the wild-type sequence may be too immunogenic for repeated use as gene delivery vehicles in gene therapy. However, due to their high immunogenicity and apparent adjuvant properties, they could be modified and used as vaccines either on their own or complexed with DNA.

Introduction

The major capsid protein (VP1) of murine polyomavirus, a small non-enveloped DNA tumour virus, is capable of self-assembling into virus-like particles (VLPs), or pseudocapsids, when expressed alone by means of a recombinant baculovirus (Montross et al., 1991). These VP1 VLPs have been shown to bind purified plasmid DNAs encoding reporter genes and deliver them into cells in vitro, resulting in the expression of the reporter transgene (Forstová et al., 1995; Krauzewicz et al., 2000b). Intranasal administration to mice of complexes of VP1 VLP and plasmid DNA encoding β-galactosidase (β-gal) results in the in vivo detection of the transgene in many different internal organs (i.e. brain, spleen, heart, lungs and kidneys) (Krauzewicz et al., 2000a). Furthermore, expression of β-gal in these animals, as established by RT–PCR, was found to be significantly more stable than in animals which received DNA alone (up to 22 weeks post-delivery vs a few weeks only). On the basis of these data, it has been suggested that VP1 VLPs may be used in gene therapy as a potential DNA delivery vehicle, even when administered by a non-invasive procedure.

There is a large body of literature on potential DNA delivery vehicles, most of which are based on live virus vectors, and their use in gene therapy (for reviews see Alemany et al., 2000; Clayman, 2000; Lesch, 1999; Verma & Somia, 1997). However, less work appears to have been carried out to assess the immune response induced by these vehicles and any resulting inherent potential drawbacks for their use, particularly in elderly and/or immunocompromised individuals. In order to assess the immunological properties of VP1 VLPs with regard to their use as a gene therapy vehicle, we have analysed both the transfection efficiency of VLP–DNA complexes in vitro and the immune response induced to both vehicle (VP1 VLPs) and transgene product (β-gal) following intranasal delivery of these complexes to mice.

Methods

Cells and plasmids. The COS-7 cell line was maintained in DMEM (Life Technologies) supplemented with 10% FCS (PAA Laboratories) and 50 IU/50 µg/ml of penicillin/streptomycin (Sigma) at 37 °C in a
humidified atmosphere of 5% CO₂. Mouse splenocyte cultures were prepared in IMDM (Life Technologies) supplemented with 10% FCS, 0.02 mM β-mercaptoethanol and 50 IU/50 μg/ml of penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. The SF9 cell line was maintained in a 1:1 mixture of TC100 and Grace’s medium (PAA Laboratories) supplemented with 10% FCS and 50 IU/50 μg/ml of penicillin/streptomycin at 25 °C in a humidified atmosphere.

The pcMV-β vector (7.2 kbp) (Clontech) is a mammalian expression vector which contains the β-gal gene cloned downstream from the CMV immediate early promoter. The pc-EGFP vector (0.2 kbp) (kindly provided by R. K. Afghan) is a mammalian expression vector derived from pcDNA3.1(+) (Invitrogen) which contains the enhanced green fluorescent protein (EGFP) gene cloned downstream from the CMV immediate early promoter. All plasmid DNAs were purified from recombinant E. coli cultures using QIAGEN plasmid midi kits.

■ Production and purification of polyomavirus VP1 VLPs. A 500 ml SF9 culture growing in a 2 l stirrer flask, at a cell density of 1.2–1.6 × 10⁸ cells/ml, was infected with a VP1 recombinant baculovirus (Forstová et al., 1995) at an m.o.i. of 10. After 3 h incubation at 25 °C, 500 ml of fresh complete medium was added and incubation continued for 72 h at 25 °C. At the end of this period, cells were harvested by centrifugation (1500 g, 4 °C, 10 min) and the cell pellet was resuspended in 10 ml of PBS and frozen at –70 °C for later processing.

For purification of VP1 VLPs, frozen SF9 cell pellets were thawed and lysed by sonication in ice seven times for 10 s with 10 s cooling intervals, also in ice. The resulting lysate was cleared by centrifugation in a microcentrifuge (9000 r.p.m., 10 min, 4 °C) and the supernatant loaded onto a two step 20–60% sucrose gradient in PBS. Following centrifugation in a SW41Ti rotor (Beckman) (217000 g, 90 min, 4 °C), the 20–60% interphase was harvested, diluted 1:5 with PBS and CsCl added to the solution (0.41739 g CsCl/g of solution). After centrifugation to equilibrium in a SW41Ti rotor (217000 g, 40 h, 25 °C), fractions (0.650 ml) were harvested from the top and analysed for the presence of VP1 by SDS–PAGE. Fractions containing empty VP1 VLPs, as determined by VP1 density (1.29 g/ml), were pooled and dialysed overnight against PBS at 4 °C.

■ Preparation of VLP–DNA complexes and in vitro transfection. COS-7 cells were seeded in 6-well plates (Nunc) at a density of 2 × 10⁵ cells per well and incubated overnight. Transfection mixtures containing VLP/cpmV-β DNA were prepared by adding 58, 29, 14.5, 2.9, 0.58, 0.29 and 0.145 μg of pcMV-β DNA to 10 μg of VP1 VLPs (DNA/VLP molar ratios of 20:1, 10:1, 5:1, 1:1.1:5, 1:10 and 1:20 respectively) in a final volume of 105 μl. After 10 min incubation at room temperature, 0.4 ml of serum-free DMEM was added to the mixture. VLP/pc-EGFP DNA mixtures were prepared as described above, but using only DNA/VLP molar ratios of 20:1, 1:1 and 1:20. DNA-only solutions were prepared by diluting the same amounts of pCMV-β or pc-EGFP DNA with PBS to 105 μl. Liposomal transfection controls were prepared with 2.9 μg of either pCMV-β or pc-EGFP DNA and DOTAP (Boehringer Mannheim) according to the manufacturer’s instructions.

For transfection, the COS-7 cell monolayers were washed once with serum-free medium and overlaid with the VLP/DNA mixtures. After 90 min incubation at 37 °C with occasional rocking, DMEM with FCS was added to a final FCS concentration of 10%. Transfections with DOTAP were carried out according to the manufacturer’s instructions. Following 48 h incubation at 37 °C, cells were washed twice with PBS, fixed with 0.1% glutaraldehyde in PBS, washed twice with PBS and finally overlaid with the chromogenic substrate [5 μM K-ferricyanide (Sigma), 5 μM K-ferrocyanide (Sigma), 2 mM MgCl₂ and 400 μg/ml X-Gal (Promega)]. The total number of blue cells in each well, after 24 h incubation in the dark at room temperature, was established by visual examination under the microscope. All transfection experiments were carried out in triplicate and statistical analysis (t test) was carried out on the data sets.

■ Immunizations. Six-week-old female barrier-reared BALB/c mice (Harlan Laboratories) were bled prior to intranasal dosing by tail snipping under local anaesthetic and tested by ELISA for previous exposure to polyomavirus VP1. ELISA-negative animals were divided into four groups, with each animal being immunized by intranasal delivery under anaesthesia with either PBS, 50 μg of VP1 VLPs, 50 μg VP1 VLPs + 3 μg pCMV-β or 3 μg PCMV-β, all in 30 μl volumes. On day 14, all animals were boosted with the same antigens and on day 28 they were killed by cardiac puncture and their spleens removed. Genital lavages were obtained from all animals before culling by rinsing the vaginal cavity twice with 50 μl PBS. Each experimental group, with the exception of the PBS group, for which n = 2, contained four animals. Experiments were carried out in duplicate.

■ Cell proliferation analysis. Mouse spleens were gently pressed through cell strainers and red blood cells removed by treatment with red cell lysis buffer (9 parts 0.16 M NH₄Cl and 1 part 0.17 M Tris, pH 7.2). Splenocyte suspensions from each experimental group were prepared at a density of 2 × 10⁶ cells/ml containing either no antigen, phytohaemagglutinin (PHA) (1 and 10 μg/ml), lysyme (3 and 20 μg/ml), VP1 VLP (1, 3 and 20 μg/ml), recombinant β-gal type VIII (Sigma) (3 and 20 μg/ml), pCMV-β DNA (1 and 3 μg/ml) or pc-EGFP DNA (1 and 3 μg/ml). These splenocyte suspensions were seeded in quadruplicate in 96-well plates (Nunc) at a density of 2 × 10⁵ cells per well. After 4 days incubation at 37 °C, 0.25 μCi of [³H]thymidine (Amersham-Pharmacia) was added to each well and plates were incubated for 6 h at 37 °C. Proliferation levels were measured for each experimental group in a β counter. The results obtained for each group from the duplicated experiments were pooled and statistical analysis (t test) was carried out on the data sets.

■ ELISA. Maxisorp 96-well plates (Nunc) were coated overnight at 4 °C with 100 μl per well of either 5 μg/ml VP1 VLPs or 1 μg/ml recombinant β-gal type VIII (Sigma), both in PBS. After removing the antigen solution, wells were coated with 200 μl of 2% milk powder in PBS. After 2 h at room temperature, the blocking solution was removed and triplicate wells were overlaid with 100 μl of decreasing tripling dilutions of the different experimental sera (starting at dilution 1/60) and vaginal (starting at dilution 1/15) samples. Following 1 h incubation at room temperature, plates were washed four times with PBS–Tween 20 (0.1%) and overlaid with 100 μl of either rabbit anti-mouse HRP-conjugated sera (Dako) or goat anti-mouse IgA HRP-conjugated ascites fluid (Sigma). After 1 h incubation at room temperature, plates were washed four times with PBS–Tween 20 (0.1%) and overlaid with 100 μl of substrate solution [0.1 mg/ml 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma) in 0.05 M phosphate–citrate buffer pH 5.0 containing a 1/4000 dilution of 30% H₂O₂]. The reaction was terminated by adding 50 μl of stopping solution (50% dimethylformamide, 20% SDS) and the absorbance of each well at 405 nm was determined.

Results

In vitro transfection using VP1 VLP–DNA complexes

In order to establish the best conditions for the efficient delivery of DNA by VP1 VLPs, COS-7 monolayers were transfected with different ratios of VLPs and supercoiled DNA mixtures. After 10 min incubation at room temperature, the absorbance of each well at 405 nm was determined.
Polyomavirus pseudocapsid–DNA complexes

Transfection efficiency of VLP–DNA complexes and DNA alone in COS-7 monolayers. The values represent the total number of blue cells observed in a 35 mm dish following transfection of COS-7 monolayers with pCMV-β either alone (▲) or complexed with VP1 VLPs (■). Plotted is the mean number of blue-stained cells ± standard error (S.E.) against the natural logarithm (LN) of the amounts of DNA used for transfection.

Immune responses to both VP1 and transgene products

The peak of transfection efficiency observed at a VLP/DNA ratio of 1:5 refers only to a short-term in vitro experiment using COS-7 cells. Obviously, this observation is no evidence for this ratio being the most appropriate for the efficient transfection of mouse tissues in vivo, but it does act as a guide. This DNA/VLP ratio was thus used to dose mice intranasally with the aim of studying the immune response to both components of the VLP–DNA complexes. Female BALB/c mice were dosed with PBS, VP1 VLP/pCMV-β DNA, VP1 VLP or pCMV-β DNA. Animals receiving VP1 VLP/pCMV-β DNA were given 50 µg of VLPs complexed with 3 µg of pCMV-β, corresponding to a DNA/VLP molar ratio of 1:5, which provided the highest transfection efficiency rate in vitro. Animals receiving VLP alone or DNA alone were immunized with the same amounts of antigen. Immunizations were carried out on days 0 and 14, and blood, vaginal lavages and spleens were collected on day 28.

Splenocyte suspensions from each experimental group were stimulated with either PHA (1 and 10 µg/ml), lysozyme, β-gal (3 and 20 µg/ml), VP1 VLP (1, 3 and 20 µg/ml), pCMV-β DNA or pc-EGFP DNA (1 and 3 µg/ml). Stimulation indices achieved by each experimental group against the tested...
Fig. 2. Stimulation indices for the experimental groups treated with different antigens. Histograms have been derived from the [3H]thymidine incorporated in the different experimental groups (as indicated at the bottom of each column) following stimulation with the experimental antigens indicated at the top of each graph.

Fig. 3. ELISA showing total serum and mucosal IgA anti-VP1 immunoglobulins. Tripling decreasing dilutions of sera and mucosal lavages from animals immunized with VP1 VLPs, VP1 VLPs/pCMV-β, pCMV-β or PBS were analysed in triplicate by ELISA against VP1 VLPs.

Antigens are shown in Fig. 2. In order to increase the stringency of these assays the positive response threshold was set at a stimulation index (SI) of 3. The average non-stimulated counts (background proliferation) of all groups in the two independent experiments carried out was $272 \pm 26$ c.p.m.

All experimental groups stimulated with PHA (10 µg/ml)
achieved an SI > 3 (minimum observed SI = 6.5). No experimental group stimulated with lysozyme achieved an SI > 3 (maximum observed SI = 1.42). For the experimental antigens, differences in the SI for each group were evident. For stimulation with VP1 VLP, only splenocyte cultures from animals immunized with either VLP–DNA or VLP alone achieved an SI value above the activation threshold of 3, but differences were observed in the response to VP1 among these two experimental groups. Animals dosed with VLP–DNA complexes reached a lower maximum SI (7.83 ± 0.50) than animals immunized with VP1 only (9.26 ± 1.45). Moreover, whilst the VLP–DNA group reached constant SI values with little evidence of titration, the VLP-only group was characterized by a reverse rate of titration. At the lowest VP1 concentration, this group achieved an SI value (9.26 ± 1.45) more than double that achieved at the highest VP1 concentration (4.27 ± 0.22). This appears to suggest that, under these experimental conditions, the VLP-only group is being over-stimulated. There is no obvious explanation for the lack of titration in the VLP–DNA group. Taken together, the data suggest that VLP–DNA complexes, although highly immunogenic, may be less efficient at inducing VP1-specific responses than VLPs on their own.

For stimulation with β-gal, only animals immunized with either VLP–DNA complexes or DNA alone achieved SI values above the activation threshold of 3. These SI values were found to titrate, with both groups achieving their maximum SI at the highest concentration of β-gal used. A major difference between these groups was that animals immunized with VLP–DNA reached a statistically significant (P < 0.05, t test) higher maximum SI (4.36 ± 0.30) than those immunized with DNA only (3.24 ± 0.13). This corresponds to a 35% increase in the β-gal-specific response.

For stimulation with pCMV-β or pc-EGFP DNA, no experimental group achieved SI values above the activation threshold of 3. The data indicate that our choice of antigen concentration was low enough so as not to induce non-specific proliferation of B-cells caused by unmethylated CpG motifs in the bacterial plasmid DNA (Holt, 1995). In addition, they also strongly suggest that DNA concentrations flanking that at which maximum COS-7 transfection is achieved in vitro (1.45 µg/ml, which corresponds to 0.58 µg of DNA) are not necessarily those at which maximum transfection of splenocytes, and by deduction other cell types, is achieved.

Regarding the humoral immune response to VP1 and β-gal, pre-dosing sera from all animals were found to be negative to these antigens by ELISA (data not shown). Following intranasal delivery, ELISA assays for total serum antibody and mucosal IgA to these antigens gave the data presented in Fig. 3. All animals immunized with either VLP–DNA complexes or VLP alone developed serum antibodies against VP1. The titre of these antibodies, despite the differences observed in the proliferative responses to VP1 among the experimental groups, was similar. Our data do not discriminate between antibody isotype, hence we cannot specify whether a differential degree of response at the IgM and IgG levels is masked by the similarity in the antibody titre. Anti-VP1 IgA antibodies were also found in the vaginal lavages, but again no differences in titre were observed between the VLP–DNA complex or VLP alone groups. This IgA response clearly shows that intranasal delivery of polyomavirus VP1 VLPs can, as shown for human papillomavirus (HPV) VLPs (Balmelli et al., 1998), induce mucosal antibody responses. No antibodies to β-gal were detected in the sera of animals immunized with VLP–DNA complexes or DNA alone.

Discussion

In this study our aims were to assess DNA transfection efficiency by VP1 VLPs in vitro, as a guide for in vivo work, and to analyse the immune response induced to both VP1 and the reporter gene product following intranasal delivery to mice of VP1 VLP–β-gal encoding plasmid DNA complexes.

Short-term (48 h) in vitro transfection efficiency analysis of VP1 VLP–DNA complexes and DNA alone showed that both systems were significantly less efficient at transfecting COS-7 monolayers than commercially available liposomal systems (DOTAP). Overall, the transfection efficiency of VLP–DNA complexes appeared to be lower than that of DNA alone, but these differences, with the exception of those observed for DNA/VLP ratios of 1:20 and 20:1, were not statistically significant. Our data indicate that murine polyomavirus VP1 VLP–DNA complexes are poor short-term in vitro transfection reagents. This observation contrasts with other reports indicating that VLPs of human polyomavirus JC (Goldmann et al., 1999) and HPV16 (Touze & Coursaget, 1998) can efficiently deliver DNA to cells in vitro. In these reports, however, DNA was encapsidated into the VLPs by a process of assembly and disassembly, whilst in our case this route is not very efficient and hence DNA was simply mixed with the VLPs. This is apparently an important difference since one report (Touze & Coursaget, 1998) clearly indicates, whilst another suggests (Ou et al., 1999), that mixing DNA and VLPs results in poor short-term in vitro transfection. Despite this, our earlier findings (Soeda et al., 1998; Krauzewicz et al., 2000a) suggest that DNA introduced by VP1 VLPs remains in the cell for longer than DNA delivered via a liposomal route. As established by electron microscopy (our unpublished observations), not all supercoiled plasmid DNA added to the VLPs appears associated with the particles; a significant proportion remains free in the preparations. Therefore, it is possible that the observed level of short-term in vitro transfection may reflect primarily transfection by free DNA.

The peak of transfection efficiency observed at a DNA/VLP ratio of 1:5 is based on a short-term in vitro experiment using COS-7 cells. This observation, whilst not evidence for such a ratio being the most appropriate for the efficient transfection of mouse tissues in vivo, does provide a guide. Consequently, this
ratio was used to dose mice intranasally with the aim of studying the immune response to both components of the VP1 VLP–DNA complexes. Our data show that murine polyomavirus VP1 VLPs are very immunogenic, inducing high cellular proliferation and antibody titres both in serum and mucosal surfaces (IgA). This observation differs with those regarding human JC polyomavirus VLPs, which fail to activate dendritic cells (Lenz et al., 2001) and to induce an immune response (Goldmann et al., 1999). We show that intranasal immunization with VLP–DNA complexes induces a lower proliferative response to VP1 than immunization with VP1 alone, but these differences are not observed in the antibody response. Differences were observed also in the immune response to β-gal. Animals dosed with VLP–DNA had higher proliferative responses to β-gal than animals dosed with DNA alone. Despite this difference in the proliferative response, no serum or mucosal antibody responses were detected against β-gal. Our data offer no explanation for the differential degree of proliferation to VP1 in the VLP–DNA and VLP-only groups. However, the increased proliferation against β-gal of the VLP–DNA group compared to the DNA-only group clearly suggests that the VP1 VLPs are acting as physically attached adjuvants in the induction of immunity. The lack of a detectable antibody response to β-gal may reflect the lag period existing between delivery of the plasmid and transgene expression and the short time between first immunization and terminal bleeding (28 days). Alternatively, it may also reflect lower levels of transgene expression and ultimately low amounts of the protein being released from the cells, an event found to be required for the development of transgene-specific immunity following DNA immunization (Ulmer et al., 1996).

Despite a higher in vitro transfection efficiency, we were unable to compare the immune response to β-gal induced following immunization with pCMV-β combined with DOTAP, since an effective preparation of the liposomal solution for the amount of DNA required could not be prepared in the 30 µl volume allowed for intranasal immunization.

Considered together, our earlier and current data suggest a possible explanation for our observations. After intranasal delivery, the plasmid DNA, either delivered on its own or present free in the VLP–DNA preparation, as well as some VLP–DNA complexes, would be taken up by the cells of the nasal mucosa, lung epithelium or by immune cells present in these tissues. Following transgene expression and antigen processing and presentation, an immune response to β-gal would be induced. In the case of the animals dosed with VLP–DNA complexes, the anti-β-gal response would benefit from the high immunogenicity and likely adjuvant properties of the VP1 VLPs, resulting in the higher SI observed. It is proposed that a proportion of the VLP–DNA complexes not processed immediately may, in contrast to DNA delivered on its own, reach the bloodstream, where they would travel for a period of time to other organs, ‘infecting’ other cell types or being processed by immune cells. This would result in a continuous stimulation of the immune system through sustained low level presentation of antigen at different sites delivered by a highly immunogenic VLP, probably also acting as an adjuvant. This first initial boost, followed by the continuous low level presentation of the antigen, may be responsible for the increased proliferative response we observe against β-gal. As suggested earlier, the lack of detectable antibodies to β-gal may be due to low in vivo transfection efficiency, the lag period existing before transgene expression and/or low level of transgene expression and extracellular release.

If this postulate is not correct and instead all VLP–DNA complexes are taken up and processed at the same time, VP1 may simply act as an adjuvant in the development of the immune response to β-gal. Indeed VLPs of other human viruses have been reported to induce strong immune responses (Kirnbauer et al., 1992; Rose et al., 1994). This option, however, does not explain the reported longer-term expression of the transgene product observed when the DNA is delivered associated with VP1 VLPs, rather than on its own (Soeda et al., 1998; Krawczewicz et al., 2000a). Furthermore, although VP1 VLPs may well act as an adjuvant, it is difficult to reconcile an adjuvant-only idea with the lack of a detectable antibody response to β-gal, since in this case a much greater number of cells would be expected to be expressing the antigen.

Neither of the above hypotheses can explain why the proliferative response to VP1 is lower in animals immunized with VLP–DNA complexes than in those that received VLPs alone. Similarly, our present data cannot account for the lack of an anti-β-gal antibody response. Further long-term experiments are required to address these questions.

In summary, our data show that murine polyomavirus VP1 VLP–DNA complexes are poor short-term in vitro transfection reagents and, as currently used with a wild-type VP1 sequence, too immunogenic for repeated use as gene therapy vehicles. A potential solution to this problem would be to use less immunogenic mutants of VP1, an alternative currently being explored. Finally, the high immunogenicity of these VLPs and VLP–DNA complexes suggests that they could be of value in vaccination, either on their own or complexed with DNA. A modified approach would involve the incorporation of epitopes encoded by the transgene in the sequence of the VLP so that, post-delivery, VLPs would provide the initial challenge and the DNA, aided by the adjuvant properties of the VLPs, the continuous low level stimulation required for long-term immunity.

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