RESEARCH ARTICLE

PEI – a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell response and FasL-mediated cell death in mice

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Polyethyleneimine (PEI) is one of the most effective gene delivery systems available today. However, very little is known about its ability to stimulate a systemic immune response and the molecular mechanisms thereof. However, this information is vital for the future development of new gene delivery systems.

Here we address this issue by studying gene expression profiles from spleen lymphocytes after *in vivo* immunization of mice with PEI formulated with a reporter plasmid (PEI+) or the formulation alone (PEI–). PEI– was found to provoke the activation of genes with important immunostimulatory functions, but without the necessary costimulatory signals. PEI+ resulted in: a mixed Th1/Th2 response; activation of both

CD8+ and CD4+ T cells, with a larger effect on CD4+; and FasL-mediated antigen-induced cell death. A comparison of the immune responses of PEI+ with that of the clinically used tetanus toxoid–aluminum phosphate vaccine showed that the DNA vaccine provoked a stronger immune response as compared to the protein vaccine. However, many genes involved in other cellular responses such as apoptosis, stress responses and oncogenesis were activated in PEI+, supporting the theory of immunostimulation by danger genes, but also pointing toward possible adverse reactions such as Alzheimer's disease.

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Introduction

The use of DNA vaccines has been ascribed a promising future, revolutionizing medical technology, and several such vaccines are currently in clinical trials.¹ Most of these trials concern vaccines based on viral delivery of DNA; however, nonviral delivery systems have recently become more popular.

Naked antigen encoding DNA are known to be able to synthesize the encoded protein and evoke a subsequent immune response of both the cellular and humoral kind, if administered by the intramuscular or intradermal routes.^{2,3} Intranasally administered naked DNA has been shown to evoke a widespread mucosal immunity in animals.⁴

Humoral and cellular immune responses have also been reported for delivery systems based on cationic lipids⁵ and cationic microparticles⁶ and a protective immune response has been observed with orally given Chitosan–DNA complexes in a murine peanut allergy model.⁷

Several other nonviral gene delivery systems have been developed hitherto, but unsolved problems remain. They include targeting to specific tissues if not administered directly to the desired tissue and improvements in all aspects of gene delivery to increase efficiency. Polyethyleneimine (PEI) is a cationic polymer, which

has been shown to be one of the most effective DNA delivery system known, both to cells in culture and *in vivo*,⁸ shuffling the DNA directly to the nucleus^{9,10} and with high-level gene reporter activity already 6 h after administration.¹¹ PEI has also been shown to be a very effective gene delivery vehicle for lung transfection, producing high antibody titers against the encoded protein.¹²

However, the use of PEI as a gene delivery system in humans is hampered by its well-known toxicity. We have therefore used PEI as positive control in a toxicogenomics analysis of polycationic gene delivery systems (manuscript in preparation). Toxicity is the reason why no clinical studies with PEI are currently performed, with the consequence that not very much is known about the types of immune responses evoked by PEI, besides its ability to activate the complement system upon intravenous administration.¹³ However, because PEI is the one of most effective gene delivery system known and because we wanted to use the information gained in the development of efficient, but safer gene delivery systems, we decided to study PEI's ability to stimulate a systemic immune response when formulated with or without a LacZ– reporter gene plasmid and administered intratracheally to mice.

Results and discussion

In this study, we used a similar experimental approach as reported earlier,¹⁴ which is a comparison between the

gene expression profiles of the adjuvant–antigen complex, here PEI formulated with LacZ (PEI+), with that of the formulation alone (PEI–) and that of non-immunized mice (N). In detail, the reporter plasmid CMVSPORT- β -gal, which encodes the gene for the LacZ protein, was formulated with PEI (PEI+). The DNA vaccine or the formulation without plasmid was administered intra-tracheally to mice at two different doses according to a standard immunization protocol, using non-immunized mice as controls for all four groups. After a booster dose, spleen cells were harvested and pooled for each formulation ($n=3$ –4 mice) to diminish interindividual variation effects.^{14–16} The spleen lymphocytes were isolated and restimulated *in vitro* with LacZ antigen peptide for 6 days and a second time for 4 and 24 h before RNA purification. These time points have been verified earlier as ideal for the study of cytokine responses on the gene expression level.^{14,16} Spleen lymphocytes of PEI– mice and the non-immunized mice were cultured for the same time periods, but not *in vitro* restimulated with antigen in order to avoid both potential non-specific and specific effects of the LacZ antigen. Also, we had observed earlier that *in vitro* restimulation of lymphocytes from non-immunized mice with tetanus toxoid antigen did not result in expression changes of genes other than those that are involved in mitotic effects.¹⁴ RT-PCR confirmation of the array results was not performed, because earlier we have found a good correlation between the gene expression results of the array used and RT-PCR results.^{14,16}

Dose-dependent effects

Mice given a 67.5 μ g dose of PEI without plasmid did not survive the administration procedure despite repetition of the experiment. This is not surprising as it is well known that PEI itself is toxic because of its ability to permeabilize membranes.¹⁷

In order to identify the optimal administration dose for the systemic immune response, PEI+ samples obtained from administration of the lower (33.75 μ g) and the higher (67.5 μ g) dose were compared concerning the expression levels of genes identified earlier to be important in the systemic immune response of the tetanus and diphtheria toxoid vaccines.^{14,16} In summary, gene expression levels from the higher dose PEI+ samples were much lower as compared to lower dose PEI+ samples (data not shown). To name the most important genes: the markers for antigen-specific CD8+ T cells, SATB1 and lung Kruppel-like factor (LKLf), the costimulatory signal B7-2 (CD86) on antigen-presenting cells, the early T-cell activation signal Syk, the T-cell activation antigens CD2 and LFA-1 α (CD11a) and CD4 receptor as well as genes involved in the cytokine regulation for both the Th1 and Th2 responses (data not shown). Therefore, samples derived from mice given the higher doses of DNA vaccine were excluded from further analyses.

Number of activated genes in global gene expression analysis

Not much is known of the systemic immune response of PEI alone or formulated with a plasmid. In fact, no articles studying their immune response reactions after administration could be found in the literature. We

Figure 1 Gene expression differences in spleen cells between mice treated with PEI+, PEI– and nontreated mice (N) after the second *in vitro* restimulation with LacZ peptide for 4 h (a) and 24 h (b). The differences are shown as self-organizing map clusters according to Tamayo's algorithm¹⁸ using the criteria for significant gene expression described in Materials and Methods. (a) A total of 143 genes were found in 12 clusters (c0–c11) containing between 3–51 genes at 4 h and (b) 260 genes in 12 clusters (c0–c11) containing between 8 and 55 genes at 24 h. Lines connecting the dots indicate the mean expression profiles; outer lines indicate the s.d. A complete list of the genes in each cluster can be obtained in the supplementary material at www.farmfak.uu.se/farm/pharmacogenomics.html.

therefore compared the gene expression profiles of PEI– and PEI+ mice spleen lymphocytes with the profiles obtained from non-immunized mice at each time point after *in vitro* restimulation by cluster analysis.¹⁸ A total of 12 clusters were obtained that contained genes with significant gene expression changes at each time point (Figure 1).

Counting the total number of genes with significant gene expression changes of all the clusters at each time point after *in vitro* restimulation with antigen, it was found that the total number was almost two times higher in the 24 h sample as compared to the total number of the 4 h samples (Table 1).

Two explanations are plausible: (1) the systemic immune response after immunization with PEI+ follows different kinetics as compared to the clinically used

Table 1 Number of genes with significant gene expression changes as assessed from Figure 2

	Total (%)	N (up)	PEI+ (up)	PEI+ (down)	PEI- (up)
4 h	143 (34)	61 (10)	52 (9)	7 (1)	32 (5)
24 h	260 (44)	55 (9)	53 (9)	22 (4)	121 (21)

The percentage of expressed genes (%) is calculated from the total number of genes on the array ($n=588$). The sum of the number of genes with significant gene expression changes from all samples does not necessarily correspond to the total number of genes, since genes can be activated in several samples at the same time.

tetanus- and diphtheria-toxoid vaccines because of the different antigens used; (2) different kinetics between the antigen–adjuvant combination and the adjuvant alone as compared to the clinically used vaccines. Therefore, the number of genes, which were up- or downregulated in each category, was counted for each time point (Table 1). This showed that the main contributor to the difference in the total number of genes between the two time points is PEI–, while the gene numbers for PEI+ vaccine did not change significantly. This is in contrast to our earlier observations derived from the analyses of the systemic immune response in spleen lymphocytes from mice after immunization with tetanus toxoid or diphtheria toxoid vaccines,^{14,16} where an early burst of gene expression during the immune response has been shown. However, only a smaller part of these genes were found to be involved in immune response reactions at 24 h in PEI–, while the major part of these genes were found to be involved in cellular processes other than immune response.

In conclusion, there is a difference in the kinetics of gene activation in the PEI+ vaccine in contrast to the early burst of gene expression in tetanus- and diphtheria-toxoid vaccines.^{14,16} This might be a characteristic of DNA vaccines in general or account for this specific antigen–adjuvant combination.

Immune response

Genes activated in PEI+ mice. Our earlier studies^{14,16} have shown that mRNA expression analysis can be used to differentiate between Th1 and Th2 response, as well as adaptive and innate immune response. Therefore, special interest was put into the analysis of gene activation involved in immune response in PEI+ mice. The expression levels of marker genes, which have been identified in the earlier studies, are shown in Figure 2a and b. See also Figure 1a, clusters c4, c5, c8 and c9; Figure 1b, clusters c0 and c1. All of these genes were expressed at several times at lower levels in non-immunized mice, as expected (data not shown).

Earlier identified cytokine markers for a Th1 response¹⁴ were upregulated (Figure 2a): $\text{Inf}\gamma\text{R}$, YB1 and Jak3, markers for interferon γ , as well as SLAP, which is necessary for TCR-driven IL-2 transcription. The activation of JunD and Stat 3 indicates an elevated level of IL-5. The levels of these markers were about two to three times higher than the levels reported earlier for the clinically used, intra-muscularly administered tetanus toxoid vaccine with aluminum phosphate as adjuvant.¹⁴ Therefore, a strong mixed Th1–Th2 response is apparent.

Figure 2 Expression of genes involved in immune response. The mRNA levels are shown as adjusted intensities for each gene as defined in the Materials and methods section. (a) Upregulated markers for the Th1 and Th2 response and (b) of the adaptive and innate immune response in spleen cells from PEI+ immunized as well as PEI– treated mice. (c) Upregulated genes involved in immunogenicity and immunostimulation in PEI– treated mice as compared to PEI+ immunized mice. Only the highest gene expression levels were shown if the gene was found both at 4 and 24 h after *in vitro* restimulation, with genes expressed at higher levels at 24 h marked by *.

Upregulated genes involved in the adaptive immune response in the presence of antigen were the monocytic LPS receptor CD14, the early T-cell activation signal Syk, the T-cell activation antigen CD2, LFA-1 α , the marker for CD8⁺ T cells, SATB1, and CD4⁺R (Figure 2b). Furthermore, the costimulatory signals B7-2 (CD86) on antigen-presenting cells and c-Jun, the POU domain associated factor 1, which is involved in T-cell antigen-dependent B-cell maturation, and Pax-5, important for committed B-cell differentiation, were upregulated.

In summary, genes indicative of a mixed Th1/Th2 response with activation of both CD8 and CD4 T cells including the important costimulatory signal B7-2 as well as B-cell differentiation were observed. Therefore, it can be concluded that the intratracheal administration of PEI+ vaccine according to a traditional immunization scheme resulted in a systemic immune response as observed in the gene expression profiles of mice spleen lymphocytes.

PEI is immuno-stimulatory in the absence of formulated plasmid DNA. As seen in Figure 2a and b, the levels of the genes involved in the Th1 and Th2 response and the adaptive immune response were activated at considerably high levels in PEI–, but these levels were still lower as compared to PEI+. This indicated an ability of PEI to activate certain immune response processes even in the absence of DNA. Therefore, the data were searched for any further evidence of PEI activation of the immune response.

The clusters in Figure 1a and b were analyzed for those showing higher upregulation in PEI– as compared to all other samples. The clusters c0, c1 and c2 in Figure 1a and c3, c6, c7 and c11 in Figure 1b were upregulated in PEI–. With one exemption (CD40L R; see below) only the clusters of the 24 h sample contained immune response marker genes at higher levels as PEI+. These genes are shown in Figure 2c.

They comprise earlier identified T-helper cell response markers¹⁴ as well as other immune response genes: Jak-3, a marker for interferon gamma, SLAP, which is found in TCR-driven IL-2 transcription, the IL-2-like cytokine IL-15, which activates proliferation and cytotoxicity of CD8⁺ memory cells in an antigen-independent way,¹⁹ the Th2 response gene IL-10 and the IL-1 receptor β . Furthermore, the following costimulatory signals were found: ICAM-1 (CD54) on antigen-presenting cells, CD22 on B cells, CD27 on B and T cells and CD38, which is found on activated T cells and known to stimulate B-cell proliferation. Upregulated genes, which are known to be reminiscent of B-cell activation, were IL-6R and CD40L R. Other genes, which are involved in immune response reactions, were activated in PEI–: CXCR-4, a chemokine receptor on CD4⁺ T cells; IL-7R, regulating the pre-B- and -T-cell growth; the adhesion molecules integrin α 7 and β 7, known to direct armed effector T cells to the site of infection; MIP1 α and MIP1 β , which are chemokines promoting Th1-dominant environments; GKLF; GCSF-R, which is involved in neutrophil development and differentiation; the macrophage mannose receptor involved in the identification of pathogens; IL-5R α on T cells, necessary for eosinophil differentiation; and several granzymes, involved in CTL responses in mice.

Many of these genes were hitherto only found activated in the presence of antigen^{14,16} and not in

systemic responses toward adjuvant only (aluminum phosphate).¹⁴ However, the absence of gene expression marking costimulatory signals for T cells together with T-cell activation suggests that a systemic immune response to PEI– is not activated. Instead it is proposed that PEI, in the absence of antigen-encoding DNA, has important immuno-stimulatory abilities, which has not been previously reported in the literature.

In order to understand the relation between all immune response genes upregulated in the PEI– sample, the genes were subjected to the bioinformatic tool PubGene.²⁰ This software extracts gene-to-gene cocitations from over 10 million Medline records and presents their relation as a network. Since this tool is only available for human genes, we translated the mouse gene symbols to human gene symbols using the database GeneCard of the Weizman Institute (<http://bioinformatics.weizmann.ac.il/cards>).²¹ A network was obtained, which contained all genes besides ITGB7 (data shown at www.farmfak.uu.se/farm/pharmacogenomics.html). Submitting the genes of the network to PubMeds MeSh Map tool (www.nlm.nih.gov/mesh), the three highest ranked returned term associations for these genes were somatomedins-blood (insulin-like growth factor 1 (IGF-1)) (score of 0.75), chemokines (0.73) and granulocyte colony stimulating factor (GCSF) genetics (0.70). The annotation to IGF-1 suggests also involvement in mitogenesis and antiapoptotic effects.²² IGF-1 has lately also been linked to the development of colorectal cancer in humans and mice.²²

This supports our hypothesis that PEI– has important immuno-stimulatory abilities, stimulating both Th1 and Th2 responses via GSCF. Further support can also be found in a comparison of the gene expression levels of markers for CD4 and CD8 in PEI–, + and PEI+. PEI– and + have comparable expression levels in both markers. Formulation and DNA together (PEI+) show expression of these markers at almost double levels at 4 h after *in vitro* restimulation with antigen.

PEI+ is superior to traditional vaccines in immune response gene activation. As stated above, some of the immune response genes were found at higher gene expression levels as compared to the clinically used tetanus toxoid vaccine. However, the literature is full of articles saying that DNA vaccines are less immunogenic than protein vaccines.²² We therefore compared the gene expression profiles of PEI+ with those of mice immunized with tetanus toxoid formulated on aluminum phosphate (TT_{ADJ})¹⁴ and non-immunized mice at each time point after *in vitro* restimulation with respective antigen. We speculated that a comparison was possible since systemic immune responses were analyzed, both types of vaccines were administered according to the same administration scheme have at different sites, and an *in vitro* restimulation with respective antigen was performed. Using the GeneCluster program²³ the comparison resulted in 12 clusters for each time point with about 25% of all genes on the array showing significant gene expression differences (Figure 3). No obvious differences in the gene expression levels and kind of genes, which are involved in immune response, were obtained comparing the profiles at 24 h after restimulation (data not shown). Therefore, only the data

Figure 3 Gene expression differences in spleen cells between PEI+, TTADJ-treated mice and nontreated mice (N). Spleen cells from PEI+ treated mice were *in vitro* restimulated by LacZ peptides for 6 days and a second time for 4 h, TTADJ-treated mice were *in vitro* restimulated by tetanus toxoid for 4 h. Self-organizing map clusters according to Tamayo's algorithm¹⁸ using the criteria for significant gene expression described in Materials and methods are shown. 167 genes were found in 12 clusters (c0–c11) containing 6–28 genes. Only clusters containing genes highly activated in (a) TTADJ and (b) PEI+ are shown. Lines connecting the dots indicate the mean expression profiles; outer lines indicate the s.d. A complete list of the genes in each cluster can be obtained in the supplementary material available at our website at www.farmacii.uu.se/...

of the gene expression profile of the 4 h samples are shown. Three clusters were found to contain 61 genes with higher gene expression levels in PEI+ (c9, c10 and c11) as compared to TTADJ, which was found with higher gene expression levels in clusters c2, c3 and c7 with a total of 42 genes.

Comparing the type of immune response genes represented in either group of genes, the PEI+ samples showed superiority in higher gene expression levels of immune response markers as well as the activation of a greater number of genes (22 genes) as compared to the TTADJ sample (10 genes). To name the most important ones, PEI+ had the highest levels of the interferon gamma markers YB1 and Jak-3,¹⁴ the IL-5 marker Stat3, and markers of the adaptive immune response such as the monocytic LPS-receptor CD14, the T-cell activation antigen CD2, LFA- α , the CD4 receptor, the costimulatory signal B7-2 (CD86) on antigen-presenting cells and the B-cell differentiation marker Pax-5. TA instead had higher levels of gene expression in the costimulatory signal for B cells, CD22, and the marker for antigen-specific CD8⁺ T cells, SATB1. The lower activation of B-cell costimulatory signals in PEI+ as compared to TTADJ is in agreement with the earlier observation that the major effector cells in mucosal immune responses consist of 80% in T lymphocytes and only 20% IgA B cells.²⁴ The most remarkable difference between these two gene expression profiles can be seen in the different types of T-cell activation mechanisms. While the higher upregulation of CD4R in PEI+ suggests that the LacZ antigens were to a large extent processed via the MHC II pathway, the higher expression level of SATB1 in TTADJ points out that

the TTADJ antigens were processed and presented more via the MHC I pathway. This is in contrast to what has been proposed for DNA vaccines, which is predominantly a CD8⁺ response,^{25,26} despite the fact that PEI+ shows higher interferon gamma marker levels as compared to TTADJ. However, the plasmid used in our immunizations does not contain CpG motifs, which are commonly considered to be responsible for the Th1 biasing effect.²⁷ Also, a comparison of the gene expression profiles in lymphocytes of mice immunized intra-tracheally with PEI+ or the plasmid only did not show any higher gene expression levels of SATB1 in the +sample (data not shown). Studies of the immune response after intra-nasal administration with cholera toxin B subunit in humans also showed that a CD4⁺ Th2 response was induced, pointing toward differences in immune responses correlating to the administration route.²⁸ Taken together, the observed lower CD8⁺-activating effects of PEI in the presence of DNA seem reasonable.

Activation-induced cell death

During memory development and effector expansion of rare antigen-specific T-cells within 0.5–2 days after antigen stimulation apoptotic processes play an important role in eliminating T cells, which are not specific for the inducing antigen—a process known as activation-induced cell death (AICD).²⁹ Several regulators are discussed in the literature:³⁰ Fas, which regulates the number of lymphocytes with TCR $\gamma\delta$, and the apoptosis inhibitor Bcl-2, which mediates the survival of primed T cells. We had observed earlier an involvement of Bcl-2 in the gene expression profiles of the clinically used tetanus vaccine.¹⁴ Analyzing the gene expression data of PEI+ at 4 h and 24 h after the *in vitro* restimulation with antigen, we found that Bcl-2 was not upregulated. Furthermore, two antagonists of Bcl-2 and members of the Bcl-2 family, BAK1³¹ and Bcl-xs,³² were upregulated. Because of these data and reports from the literature that apoptotic processes are regulated by competitive dimerizations between different agonists and antagonists,³³ it is proposed that an upregulation of Bcl-2 is not responsible for the survival of antigen-specific T cells in spleen lymphocytes, when mice were immunized with PEI+.

Instead, we found that PEI+ activated the expression of FasL, an apoptosis promoter, which is involved in cytotoxic T-cell-mediated apoptosis and in the antigen-stimulated suicide of mature T cells.^{30–31} FasL together with TCR is also known to induce the expression of TRAIL,³⁶ which was also found upregulated in PEI+. To summarize, the data show that it is likely that FasL mediates the activation-induced cell death in spleen lymphocytes, isolated from mice immunized with PEI+ after intra-tracheal administration.

In conclusion, we propose that different vaccines, or different antigen–adjuvant combinations, activate a different type of regulator during activation-induced cell death. Currently, three types are known: We found Bcl-2 as an inhibitor of cell death in primed T cells activated in the clinically used tetanus toxoid aluminum phosphate vaccine.¹⁴ Mitchell *et al.*³⁷ showed that the survival factor Bcl-3 is upregulated in lymphocytes from mice that had been given either LPS or vaccinia virus. Here in this study, the FasL-mediated death pathway for removal of

naïve T cells is activated in lymphocytes of mice that were immunized with PEI+.

We further speculate that the factor governing the type of regulator for activation-induced cell death (AICD) is the way the antigen is presented: Pronounced CD8⁺-mediated immune responses with inflammatory characteristics activate the Bcl-2 survival pathway as we reported earlier for the tetanus toxoid vaccine.¹⁴ Immune responses mediated via predominantly CD4⁺ instead control AICD via FasL as observed in this study (PEI+) and as reported earlier.³⁸ Finally, vaccines consisting of other types of adjuvants, such as lipopolysaccharides, induce the Bcl-3 survival pathway.³⁷ Since it was reported by Rebello *et al*³⁹ that IL-4 deprivation leads to induction of Bcl-3 expression, it can be assumed that all IL-4 affecting adjuvant and antigen-adjuvant combinations induce Bcl-3-mediated survival of primed T cells. The data presented here also suggest that the mechanisms governing immune responses are much more complex and can differentiate between different antigens and adjuvants in a much more sensitive way than recognized earlier.

Genes involved in other cellular processes

As stated above, 121 genes were activated in PEI– at 24 h after *in vitro* restimulation with antigen peptides (Table 1). Only a small fraction of these genes (a total of 23 genes or 18%) could be linked to immunological responses. In an earlier study, which compared the gene expression profiles of mice spleen lymphocytes after administration of the clinically used tetanus toxoid formulated with aluminum phosphate as adjuvant or the adjuvant alone, we had observed that adjuvant without antigen present could activate 47 genes associated with inflammation, apoptosis, stress and oncogenesis and it was proposed that these genes could be part of danger signals of the innate immune response.¹⁴ Therefore, we wanted to analyze if the large number of genes, which were activated in PEI– and suppressed in PEI+, also could be associated with the danger model.

After clustering the gene expression data (Figure 1) genes activated in PEI– were found in the clusters c1 and c2 of the 4 h sample (Figure 4a) and in the clusters c3, c6, c7 and c11 for the 24 h sample (Figure 4b). At a first glance, the over-representation of receptor expression (a number of 24) in c3 but also c6 and mainly c7 of the 24 h sample was obvious. However, the receptors seem to be involved in different cellular processes. In order to understand the combined functions of the receptors and the other genes activated by PEI–, we used the literature association tool PubGene²⁰ (see above). Since it has been proposed earlier that clusters are reminiscent of co-regulated genes involved in the same processes⁴⁰ and the entry amount of data is limited, we subjected the gene list of each cluster to the PubGene tool.

The obtained networks from each cluster were examined for participation in the literature network and nonparticipating genes were excluded (66 genes out of 139 genes). The literature subnetworks for each cluster may be searched at our website at www.farmfak.uu.se/farm/pharmacogenomics.html. The remaining 73 genes were submitted to the MeSh Map tool. The four returned term associations with the highest scores for these genes were serotonin genetics (1.00), protein kinase C genetics (1.00), glutathione transferase genetics (1.00) and Alzhei-

mer Disease genetics (0.91). The relations between serotonin and protein kinase C to the neurodegenerative Alzheimer Disease are well known in the literature, while an involvement of glutathione transferase has not been established.

It has been speculated that free PEIs exhibit toxic effects on cells because of their ability to permeabilize plasma membranes.^{41,42} Alzheimer's disease is known to be associated with the formation of fibrils from mutant amyloid proteins. In a recent publication it was found that these fibrils are very similar to pore-forming toxins and the authors suggest that inappropriate membrane permeabilization is the cause of cell death and dysfunction in Alzheimer's disease.⁴³ Godbey *et al*⁴⁴ state that free PEI leads to growth inhibition and cell death and that removal of free PEI by formulation with DNA decreases toxicity.

Taken together, PEI– activates genes involved in cellular processes such as cell cycle regulation, oncogenesis and differentiation, indicating toxic characteristics of free PEI and the danger of adverse reactions after immunization with PEI-containing vaccines. This is supported by the fact that mice in our study given the higher dose of PEI without DNA plasmid present died shortly after the booster administrations.

Comparing the gene expression profiles in mouse spleen lymphocytes caused by PEI– administration to lung mucosa and the one caused by aluminum phosphate administration to muscle, we were not able to detect many similarities, beside four genes, which can be associated with immune response reactions: TFIID, identified as possibly involved in immune responses in one of our earlier studies;¹⁶ SLAP, which is necessary for TCR-driven Il-2 transcription; the GCSF receptor, necessary for the production of granulocytes, macrophages and dendritic cells; and cathepsin B, involved in antigen processing in endocytic vesicles. Obviously, each adjuvant elicits different systemic responses involved in immune response or other cellular processes, but this could be partly caused by the different administration routes, i.m. for aluminum phosphate and i.t. for PEI. This is in line with our earlier observation that each adjuvant-antigen combination activates a different gene expression profile in a systemic response.¹⁶

However, one similarity to the tetanus vaccine could be found. This is the down-regulating effect of antigen—in the case of PEI–LacZ formulation encoded in the plasmid—on the expression levels of those genes, which were highly upregulated if only the adjuvant is administered (Figure 4, Figure 1a clusters c1 and c2, Figure 1b clusters c3 and c7). Since the protein encoded in the DNA plasmid is not present at the time of administration in contrast to the tetanus vaccine, it is concluded that suppressive effects of the antigen are not exerted at the time of administration but during the presentation of the antigen in immune response reactions.

This supports our earlier proposal that adjuvant elicits responses which are part of the endogenous danger signals in the innate immune system and that this modulation by the antigen is necessary to protect against adjuvant-mediated effects.¹⁴ This greatly supports the danger theory postulated by Matzinger *et al*.²³ It also appears to be a general mechanism applying to all types of antigens in vaccines, protein and DNA, studied by us so far.^{14,16}

Obviously, this adjuvant-modulating capacity of antigens is of great importance for the safety of vaccines and protection against possible unwanted adverse reactions caused by the adjuvant. However, it is not clear if the adjuvant-modulating capacity of antigens is not only coupled to the presentation of the antigen but also to the strength of the antigen's immunogenicity. If this is the case, the often poorly immunogenic antigens produced

by recombinant techniques present an obvious danger for adverse reactions. In the case of DNA vaccines, the danger lies in the time interval between the delivery of the DNA and the synthesis/presentation of the antigenic protein. Therefore, gene delivery systems with rapid onset of gene expression and translation are preferable as is the case with PEI. Further research of this interplay is needed to develop new, safe and more effective vaccine adjuvants.

Figure 4 Clustergram of the gene expression profiles from the six clusters c1 and c2 in Figure 1a (a) and c3, c6, c7 and c11 in Figure 1b (b) obtained using the Treeview program.¹¹ The sample is indicated at the top of each column. Red indicates upregulated and green downregulated genes, respectively. (c) Functional categories of genes significantly activated in PEI-. The information obtained by clustering (Figure 1) was used to assess the involvement of the genes in different cellular processes as specified by the array manufacturer. The percentage of each category compared to the total number of activated genes is shown. The cells were cultured for 4 h (A) and 24 h (B).

Figure 4 Continued

Materials and methods

Animal experiments

The animal experiments were approved by The Swedish National Board for Laboratory Animals (the local ethical committee). Male Balb/c mice, 8–10 weeks old (Charles River), were randomly separated into groups of three to four animals. The mice were given an intra-tracheal dose of DNA vaccine containing 50 or 100 µg of endotoxin-free LacZ plasmid (pCMVSPORT.β-gal, Invitrogen) (obtained from Aldrevon) formulated with 33.75 or 67.5 µg PEI polymer (PEI+) 25 kDa (Sigma) at a charge ratio of 5:1/-/. Other groups of mice were given the same doses of PEI without plasmid (PEI-). The same doses were administered 2 weeks later. At 3 weeks after the booster, spleens were isolated, pooled for each group to compensate for individual genetic variation, and washed in cold PBS.

Cell culture experiments

A single-cell suspension from the spleens was prepared and erythrocytes were removed by incubation in 1 M Tris

buffer, pH 7.2 on ice for 10 min. The cells were washed twice with RPMI medium (Dulbecco) supplemented with 292 µg/ml L-Gln, 50 µM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated FCS (Gibco). Aliquots of the cells (1.25×10^6 cells/ml) were plated into cell culture flasks and incubated for 6 days, at 37°C in 5% CO₂ at approximately 90% humidity with the antigen peptide for LacZ beta-galactosidase peptide₈₇₆₋₈₈₄ (TPRPARIGL; 99% purity) (obtained from the Core Facility for Protein/DNA Chemistry, Queens University, Kingston, Canada) at a dose of 1 µg/ml cell culture medium, followed by a second incubation with the same peptide for 4 or 24 h, respectively. The control groups of mice, which had received PEI alone, were cultured but not *in vitro* restimulated with peptide antigen. The *in vitro* restimulation with pure tetanus toxoid (GMP grade, SBL vaccine, Solna, Sweden) was performed as reported earlier.^{14,16} After restimulation, the cells were washed with cold PBS and used for RNA isolation.

Analysis of gene expression by array technology

Total RNA was purified from splenocyte cells using the Ambion's Totally RNA™ kit. DNA contamination was removed using DNase I (Promega). Atlas mouse cDNA expression arrays with 588 genes (Clontech) were used according to the manufacturer's guidelines and the reproducibility was assessed as reported earlier.¹⁴ The level of expression was quantified by phosphorimaging (Packard) and the obtained image data were processed using the AtlasImage program version 1.01a (Clontech). Signals lower than the average background signal were filtered out. The adjusted intensity of each gene was obtained by subtracting the average background signal from the signal intensity. To compare two or more arrays, the adjusted signal intensities of all genes on the arrays were normalized using the global mode and the sum method. Briefly, the ratio of the adjusted signal intensity of gene Z on array 1 to the adjusted intensity of the same gene Z on array 2 was calculated for all the genes on the array and averaged. In order to filter for genes with significant gene expression changes, we used the following criteria: only genes with a difference in signal strength of more than 100 and a more than two-fold expression difference as compared to nontreated control mice in at least one sample were included.^{14–16} Genes were sorted by the program Excel, clustered by the program Genecluster¹⁸ and visualized by Treeview.⁴⁵ Other bioinformatics analyzers used included GeneCard (<http://bioinformatics.weizmann.ac.il/cards>),²¹ PubGene²⁰ and MeshMap (www.nlm.nih.gov/mesh).

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