

Tetanus antigen modulates the gene expression profile of aluminum phosphate adjuvant in spleen lymphocytes *in vivo*

K Regnström
EGE Ragnarsson
N Rydell
I Sjöholm
P Artursson

Department of Pharmacy, Uppsala University,
Uppsala, Sweden

Correspondence:
K Regnström, Department of Pharmacy,
Uppsala University, Box 580, SE-75123
Uppsala, Sweden
Tel: +46 471 4628
Fax: +46 471 4223
E-mail: Karin.Regnstrom@farmaci.uu.se

ABSTRACT

Adjuvants play an important role in stimulation of the immune response to antigens. Very little is known about the molecular mechanisms of this stimulation. Here we address this issue by studying gene expression profiles from spleen lymphocytes after *in vivo* immunization of mice with a clinically relevant vaccine, tetanus toxoid formulated with aluminum phosphate as adjuvant (TT_{ADJ}), or the adjuvant alone (ADJ). The Th1/Th2 response to TT_{ADJ} was obtained from a combination of up- and downstream markers to conventional cytokines, which were in good agreement with cytokine protein levels. A clustering algorithm revealed that ADJ elicited expression of 47 genes active in cytotoxic lymphocytes, inflammation, oncogenesis, stress, toxicity and cell cycle regulation. In TT_{ADJ} these adjuvant-elicited genes were expressed at lower levels and a compensatory onset of protective and inhibitory genes was observed. We conclude that the antigen, to a larger extent than previously recognized, modulates the molecular mechanism of the aluminum phosphate adjuvant and that the identified genes may serve as predictive biomarkers in the development of new adjuvants and vaccines. *The Pharmacogenomics Journal* (2002) 2, 57–64. DOI: 10.1038/sj/tpj/6500080

Keywords: vaccine; tetanus toxoid; antigen; adjuvant; aluminum phosphate; cDNA array

INTRODUCTION

Modern vaccines elicit protective immunity not only by presenting antigens to the adaptive immune systems, but also by stimulating the innate immune response to the antigens by immunostimulatory components included in the vaccine, known as adjuvants. Adjuvants are included since modern vaccines are based on specific antigens. Often, these are poorly immunogenic by themselves as compared to traditional killed or attenuated whole-cell vaccines, which also contain invariant stimuli for the innate immune system (such as lipopolysaccharides (LPS) and teichoic acid).¹ Unfortunately, many adjuvants are too potent immunostimulators and cause unwanted side effects.² It is therefore not surprising that essentially all clinical experience has been obtained from the traditional aluminum-based adjuvants, which have been used in humans since the 1930s.³ The immunostimulatory properties have not been fully characterized even for these aluminum-based adjuvants and undesired side effects have been reported for the administration site including erythema, subcutaneous nodules, contact hypersensitivity and granulomatous inflammation.⁴ Recently, massive accumulation of aluminum in macrophages of muscle previously injected with aluminum-based adjuvants was associated with muscle ailment.⁵ To our knowledge, the immunological and potential side effects of the aluminum phosphate adjuvant on a lymphoid tissue distant from the administration site have not been thoroughly investigated. Such studies would give new information regarding the immunomodulatory and potential toxicological effects of the adjuvant.

Received: 22 June 2001
Revised: 14 November 2001
Accepted: 16 November 2001

When assessing the immunostimulatory effects of various vaccines, traditional protein detection methods based on immunochemical techniques such as ELISA have been used. However, these methods are capacity limited and therefore give an incomplete picture of the overall *in vivo* response to a vaccine.⁶ We hypothesized that differential gene expression analysis of spleen cells from mice immunized with a clinically relevant tetanus vaccine (TT_{ADJ}), or with the aluminum adjuvant (ADJ) alone, would give us a better view of the global gene response to a vaccine and allow us to distinguish between vaccine- and adjuvant-regulated gene responses.

In summary, the immunological response profile obtained by cDNA array technology agreed with results obtained with traditional cytokine-ELISA-assays. The results also suggested potential surrogate markers for the Th1/Th2 cytokine analysis, making it possible to rapidly map the Th1/Th2 response to aluminum-based vaccines. Further, differential gene expression analysis distinguished vaccine- and adjuvant-mediated gene regulation both with respect to the immune response and with respect to other pharmacological responses. ADJ induced many markers, which indicate a systemic toxicity. These toxic characteristics of the ADJ were suppressed in the presence of antigen (TT_{ADJ}).

RESULTS

Our experimental approach is outlined in Figure 1. Briefly, TT_{ADJ} or ADJ were administered i.m. to mice according to a standard immunization protocol, using non-immunized mice (N) as controls in both groups. After a booster dose, spleen cells were harvested and their RNA isolated for use in gene expression analysis. In experiment a, cells were isolated and restimulated *in vitro* with pure tetanus toxoid antigen (TT) for the times indicated before RNA purification. Non-immunized mice were also restimulated to eliminate the possibility that they had been exposed to the antigen before the onset of this study and to eliminate potential

non-specific *in vitro* effects of the antigen from the gene expression analysis. In experiment b, the ADJ samples were not restimulated *in vitro* with TT in order to avoid both potential non-specific and specific effects of the TT antigen. All TT_{ADJ}-immunized mice developed strong serological titers against TT.

Global Gene Expression Analysis

Fifty-one or 8.7% of the 588 genes on the cDNA array significantly altered expression (as defined in the Methods section) 4 h after TT-restimulation, while the corresponding value at 24 h was 26 genes or 4.4% (Figure 1). Thus, there is a massive change in the mRNA pool in the spleens early after TT-antigen stimulation, as previously seen for lymphocytes stimulated with superantigen.⁷ In the ADJ group, 67 or 11.4% of all genes on the array displayed altered expression. The quality of the gene expression data was assessed by repetition of the array analysis. Only 0.34% of the data had significant differences in their gene expression level as defined in the Materials and Methods section. Plotting the raw data against each other (Figure 2) according to the comparisons outlined in Figure 1 showed that the ADJ group displayed the largest deviation from the base-line gene expression in the spleen cells from control mice (Figure 2c). The majority of the genes with altered expression in the ADJ group were downregulated.

RT-PCR

In order to confirm some of the array results we performed RT-PCR for five genes, Pax-5, SATB1, LFA-β, cyclin D1 and TRAIL. All of the genes displayed gene expression levels comparable to those of the Atlas array. Thus five out of five or 100% of the genes on the Atlas array were confirmed with RT-PCR, which is higher than an earlier reported correlation of 72%.⁸

Vaccine-specific Gene Expression Profile

To validate our array technology as a tool to study Th1 and Th2 markers and to find suitable immunological markers, we compared the gene expression profiles of spleen cells from TT_{ADJ} to N. The gene expression levels of selected markers were confirmed by RT-PCR as indicated in Figure 3 (data not shown).

In order to find the time-points for maximal cytokine gene expression, mouse spleen cells were analyzed for mRNA expression of selected cytokine markers at the time points 4 and 24 h after *in vitro* TT restimulation (see below).⁹ Supernatants of TT_{ADJ} were assessed by cytokine ELISA for the two Th1 markers IFN-γ, IL-2, as well as the two Th2 markers IL-4 and IL-5 at 24 and 48 h after TT restimulation. Only one cytokine, IL-4, but not the cytokines IFN-γ, IL-2 and IL-5, are included in the Atlas cDNA array. Therefore, several up- and downstream regulators to the missing cytokines were identified and used in the comparison. The IFN-γ receptor, YB1¹⁰ and Jak-3 tyrosine-protein kinase, were chosen as IFN-γ markers. They are all upregulated in the presence of IFN-γ.^{11,12} The chemokine receptor CCR1, which is upregulated by IL-2,¹³ was chosen as IL-2 marker. As mark-

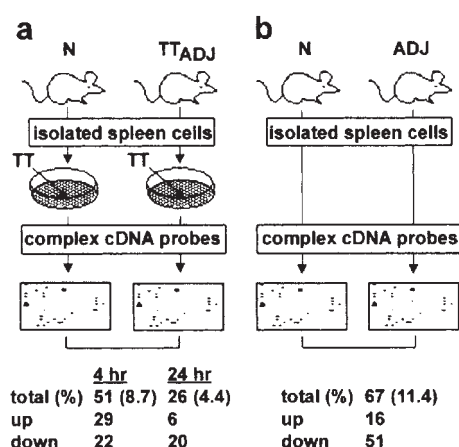


Figure 1 Experimental set-up and number of genes with significant gene expression changes. The percentage of expressed genes (%) is calculated from the total number of genes on the array ($n = 588$).

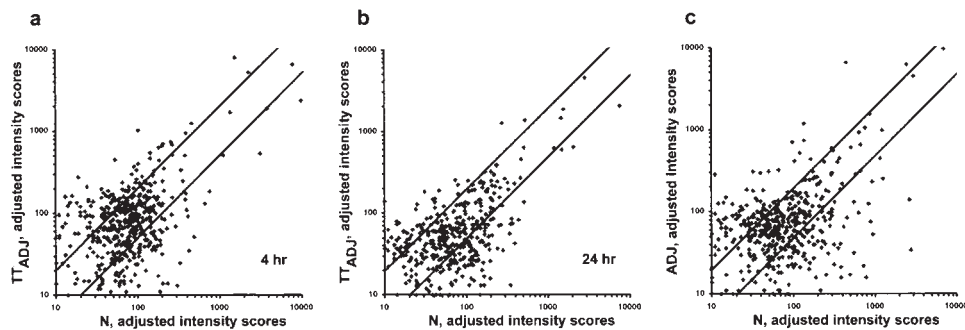


Figure 2 Global gene expression analysis. RNA was isolated and transformed to radioactively labeled cDNA as described in the Materials and Methods section. The levels of cDNA of the different genes were measured and processed by Clontech Atlas technology and by Spotfire software. The plots compare the expression levels of all genes of each pair of arrays, expressed as adjusted intensity scores as described in the Materials and Methods section. The lines represent differences of 2-fold between the two samples. Comparisons of TT_{ADJ} with N at (a) 4 and (b) 24 h after *in vitro* restimulation with TT. (c) Comparison of ADJ mice with N.

ers for the Th2 response IL-4, GATA-3, a transcription factor upregulated during Th2-specific cytokine expression,¹⁴ and IL-10 were selected. Furthermore, Jun D, Stat3 and Stat5a were selected as markers for IL-5 presence.^{15,16}

In general, the gene expression profiles obtained from the arrays agreed well with the cytokine responses (Figure 3a and b). Thus, upregulation of gene markers representative for Th1 differentiation corresponded well with the protein levels of IFN- γ and IL-2. Similarly, gene markers for an IL-5 response were clearly upregulated on the arrays, which agrees with the ELISA result obtained from the IL-5. In summary, the results obtained by both methods agreed well with each other and clearly indicated that a mixed Th1/Th2 response was obtained. Importantly, this is the expected response for a tetanus aluminum vaccine in mice.¹⁷ We conclude that the Th1/Th2 profile of TT_{ADJ} can be predicted from mRNA profiles obtained by cDNA expression arrays, using a panel of unconventional cytokine markers.

Comparison of the expression profiles between TT_{ADJ} and N also showed upregulation of several other genes involved in the adaptive immune response (Figure 3c). These genes include the monocytic LPS receptor CD 14, the early T-cell activation signal Syk,¹⁸ the T-cell activation antigens CD2 and LFA-1 α (CD11a), the T-cell adhesion marker CD44 and the markers for antigen-specific CD8⁺ T-cells, SATB1 and lung Kruppel-like factor (LKLf). Co-stimulatory signals such as B7-2 (CD86) and ICAM-1 (CD54) on antigen presenting cells¹⁹ and tumor necrosis factor receptor 2 (TNF-R2), which triggers cell death induced by T-cell activation, were also upregulated as were POU domain associated factor 1 (POU1) and A-myb proto-oncogene (which are involved in T-cell antigen dependent B cell maturation), the co-stimulatory signal for B cells, CD22; and early B cell factor (EBF) and Pax-5, which are important for committed B cell differentiation.²⁰ Many other genes involved in immunological and other cellular processes changed expression in TT_{ADJ}. A selection of these genes are discussed below. Taken together, our findings reveal the complexity of the cellular response to a vaccine and show that many proto-oncogenes and transcription factors are involved that have not yet been associ-

ated with this response in the scientific literature. (For more information see supplementary material at www.nature.com/tpj/index.html).

Adjuvant-specific Gene Expression Profile

As expected, no traces of antigen-specific T cell activation were found after ADJ administration and only a few genes involved in the immune response were upregulated by ADJ. These genes are mainly involved in Th1 responses (Figure 3d) and therefore very different from the genes activated by TT_{ADJ}. Many of the latter were downregulated in ADJ. We found that SLAP, which is required for TCR-driven IL-2 transcription, as well as the IL-2 receptor γ -subunit (IL-2 R γ) which is part of several cytokine receptors, were upregulated by ADJ. The same was observed for Cas, which is a signal transducer in integrin-mediated cell adhesion, and relaxin, which is involved in Th1 differentiation. There were also genes, where ADJ had a downregulating effect on the expression. Most of them are not known to be involved in the immune response, but two, Stat1 and prothymosin α are known to be involved in T cell proliferation (data not shown). Furthermore, we found YB1, Jak3, Stat6, the T cell activation gene CD2, the T cell adhesion marker CD44 and the CD4 receptor downregulated, all of them immune response markers upregulated in TT_{ADJ}. However, we found upregulation of genes involved in cytotoxic T cell response, CTLA-4 and LFA-1 β , and of the inflammation markers c-Jun and c-Fos.

In comparison to the TT_{ADJ}-group, we observed a remarkable difference in the activation of genes involved in apoptosis, stress, toxicity and oncogenesis. Since ADJ is also a component of the TT_{ADJ} we decided to investigate the gene expression in these categories in immunized mice.

Gene Expression Differences Between ADJ Mice and TT_{ADJ} mice

Since the control groups in the TT_{ADJ} and the ADJ experiments differed in that the former were restimulated by the TT-antigen *in vitro*, we first compared the gene expression profiles of the two controls. Apart from a few genes showing

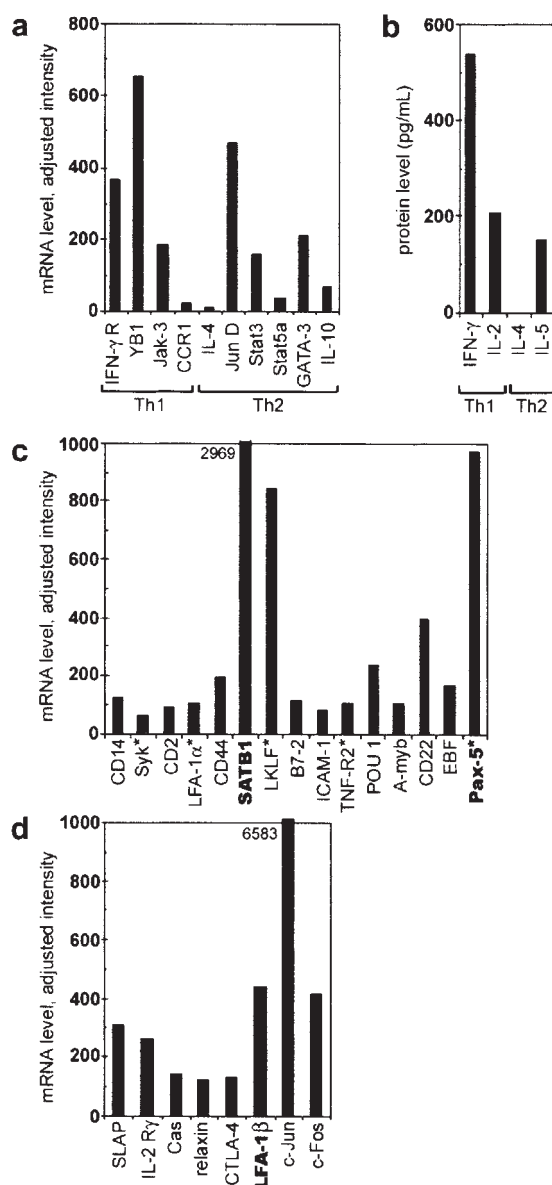


Figure 3 Expression of genes and proteins involved in immune response. The mRNA levels are shown as adjusted intensities for each gene as defined in the Materials and Methods section. (a) Selected upregulated markers for the Th1 and Th2 response in spleen cells from TT_{ADJ}-immunized mice and (b) corresponding protein levels (pg ml⁻¹) of cytokines involved in the Th1 and Th2 response. (c) Early (4 h) and late (24 h; indicated by *) immune response genes in spleen cells from TT_{ADJ}-immunized mice. (d) Upregulated genes involved in immunogenicity and in inflammation in spleen cells of ADJ-treated mice. Gene expression confirmed by RT-PCR are written in bold.

mitogenic effects, no significant global changes were observed between the two control groups (data not shown). This allowed direct comparison between the TT_{ADJ}, the ADJ and the N group. Using the GeneCluster program,²¹ as many as 203 genes, or 34.5%, had significant expression changes in at least one of the samples. This is a higher number than

the total number of genes shown in Figure 1, which can be explained by the larger number of comparisons and their greater extent of variation. This leads to more genes fulfilling the criteria for significant gene expression change as defined in the Methods section. The data were sorted into six clusters according to their expression profiles in the different samples (Figure 4a) and visualized by the Treeview program²² (Figure 4b). This second analysis confirmed our findings concerning the genes involved in adaptive immune response as well as inflammation (data not shown).

More apoptosis-inducing genes were upregulated in ADJ than in TT_{ADJ}, for example TRAIL and Alg-2, which are induced by TCR and Fas,²³ and Fas I R. We also found the pro-apoptotic Bax membrane isoform α , B-lymphocyte kinase, and the activator of apoptosis harakiri upregulated in ADJ. In contrast, Bcl-2, an apoptosis suppressor, was upregulated in TT_{ADJ}, but not in ADJ. Several more heat-shock genes (eg HSP60) and other stress response genes were activated primarily in ADJ, but some were also found activated in TT_{ADJ} mice (eg GRP78).

Many proto-oncogenes were activated by ADJ, some of them known for their involvement in differentiation during the immune response, some with unknown function. Some cancer markers, such as cyclin D1,²⁴ were also strongly induced by ADJ. In contrast, TT_{ADJ} upregulated cancer protection genes (RB1, Pml, TGF- β R, p19ink4 and p27kip1), which were downregulated in ADJ, and some cell cycle regulating genes, but also other proto-oncogenes (ROR- α -1, OCT3, SHC-transforming protein).

In conclusion, both ADJ and TT_{ADJ} changed the expression of genes with many different functions, ranging from immune response to apoptosis, stress and toxicology response to differentiation. A larger number of genes involved in the adaptive immune response were induced by the TT_{ADJ} vaccine as compared to ADJ, while most of the 48 genes upregulated by the aluminum adjuvant have functions in inflammation, stress, apoptosis, oncogenesis and differentiation. Many of these genes have not previously been associated with an immune or vaccine response.

DISCUSSION

This is the first investigation of gene expression profiles induced by the components of a traditional vaccine after immunization of a mammal. Here we discuss some of our more important observations of differential gene expression changes after vaccine and adjuvant administration and the significance of our findings for vaccination and vaccine development.

Immunogenicity

Our observations indicate that mRNA expression analysis can be used to differentiate between a Th1 and a Th2 response, in agreement with our results obtained by ELISA. In addition, these observations show that the cDNA arrays gave a more detailed picture: as expected, many genes involved in the adaptive immune response were only expressed in the presence of antigen. However, many genes, including transcription regulators for the Th1 response,

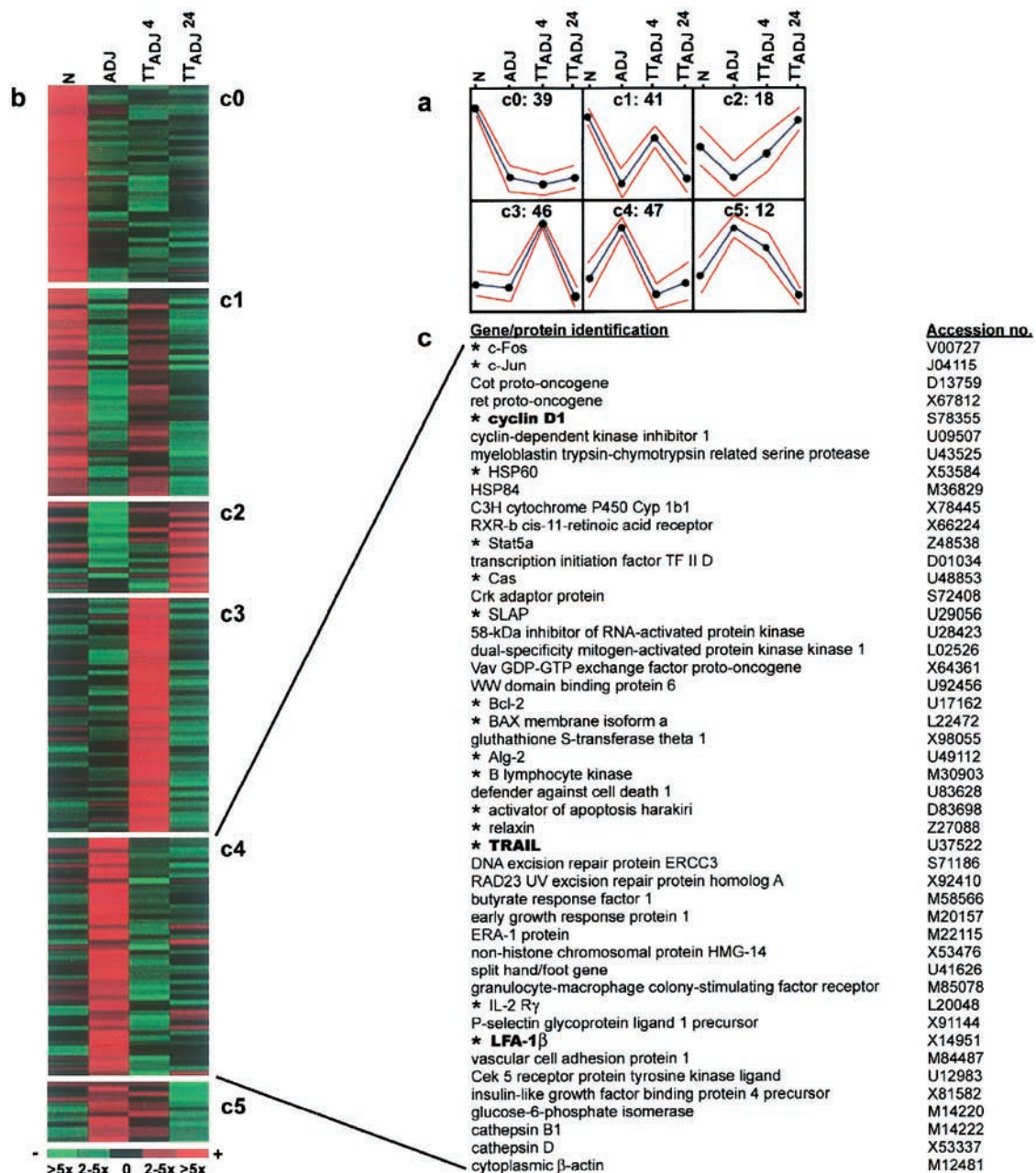


Figure 4 Gene expression differences in spleen cells between TT_{ADJ}, ADJ-treated mice and non-treated mice (N). Spleen cells from TT_{ADJ}-treated mice were *in vitro* restimulated by TT for 4 h (TT_{ADJ} 4) and 24 h (TT_{ADJ} 24). (a) Self-organizing map clusters according to Tamayo's algorithm²¹ using the criteria for significant gene expression described in Materials and Methods. 203 genes were found in six clusters (c0–c5) containing 12–47 genes. Blue lines indicate the mean expression profiles; red lines indicate the SD. A complete list of the genes in each cluster can be obtained in the supplementary material at www.nature.com/tpj/index.html. (b) Clustergram of the gene expression profiles from the six clusters in (a) using the Treeview program.²² The sample is indicated at the top of each column. Red indicates upregulated and green downregulated genes, respectively. (c) List of genes, which were highly upregulated by ADJ, as revealed from cluster c4 in (a). Genes with * are reported in the Results and Discussion sections; genes in bold were confirmed by RT-PCR.

were mainly regulated by the aluminum phosphate adjuvant on its own. These genes were activated to a much lower extent or not at all activated in the presence of antigen. The upregulation of the IL-2 R γ by ADJ does not indicate T cell activation, but T cell expansion.²⁵ We found co-stimulatory

signals for T cell activation only in the presence of antigen. These signals were the upregulation of the CD11a subunit of LFA-1 α in T cells as well as of B7-2 and ICAM-1. Without antigen present, however, the homologous cytotoxic T-lymphocyte antigen (CTLA-4) was upregulated. This is an

inhibitory signal for T cell activation,²⁶ responsible for the inhibition of the ras pathway as well as of IL-2 production.²⁷ Furthermore, CD18, an LFA-1 β component involved in leukocyte-rolling and strongly induced by pro-inflammatory cytokines,²⁸ was expressed in the ADJ-treated group. We conclude that the presence of antigen is necessary for T cell activation and, importantly, that it governs the effect of the adjuvant on the antigen-presenting cells.

Inflammation

It has been known for quite some time that administration of aluminum adjuvants coated with antigen leads to the formation of inflammatory foci that attract immunocompetent cells to the site.²⁹ The effects on the secondary lymphoid organs, however, have not previously been described.

Our results show that administration of ADJ induces the transcription of inflammatory markers also in lymphoid organs that are distant from the administration site. Thus, the immediate early c-Jun (14.8 times) and c-Fos genes (4 times) (Figure 3d) were induced. They have been described earlier as inflammatory disease-related genes in monocyte cell lines, expressed upon activation with LPS.³⁰ Substantial c-Jun expression is also observed in sub-lethal oxidative stress and toxic reactions, while c-Fos is only moderately upregulated.³¹

We speculate that the altered expressions of these and other markers are related to the reported side-effects of aluminum adjuvants. An upregulation of c-Jun, but not of c-Fos has been reported during CD28 activation,³² but it needs the additional activation of the TCR-triggering pathway not observed in ADJ to be indicative for T cell activation.³³ It is, therefore, more plausible that the effects seen indicate inflammation. Surprisingly, in the presence of antigen, c-Jun and c-Fos were expressed at the same level as in the control group (N). It seems that antigen presence is necessary to suppress an inflammatory process.

Taken together, our observations suggest that the immunostimulatory effect of the aluminum phosphate adjuvant is based on the stimulation of the IFN- γ pathway and probably also on a coupling to a general inflammatory response via c-Jun and c-Fos.

Apoptosis

Apoptosis is a part of the adaptive immune response in the antigen-induced cell death (AICD), which is involved in memory development and effector expansion of rare antigen-specific T cells within 0.5–2 days after antigen stimulation.³⁴ The identity of the mediator for this process has been discussed in the literature, and two candidates have emerged: Fas, which regulates the number of lymphocytes with TCR $\gamma\delta$ ³⁵ and the apoptosis inhibitor BCL-2. Our array data do not support an involvement of Fas in the immune response to TT_{ADJ}, since it is downregulated in the TT_{ADJ} sample and upregulated in ADJ. This rather supports the finding by Suzuki and Fink³⁶ that Fas exhibits co-stimulatory signals for the antigen-specific proliferation of CD8⁺ T cells. It also corroborates our hypothesis that the TT antigen suppresses the ADJ-induced inflammation.

The strong upregulation of BCL-2 in TT_{ADJ} points toward an involvement in the survival of antigen-activated T cells, which could be mediated via Cdk2 and caspases during the S phase of the cell cycle.³⁷ We made additional observations, which support the hypothesis, namely an upregulation of c-Myc and of cyclin D3.³⁵ We suggest that Bcl-2 is activated in TT_{ADJ} to promote antigen-activated T cell survival. An involvement of Bcl-3 in this process via an AP-1 induction³⁸ seems less plausible here, since the AP-1 factors c-jun and c-fos were downregulated in TT_{ADJ}. Since Bcl-3 can be induced by administration of LPS to mice,³⁹ one can speculate that there are several signaling pathways for T cell survival depending on the type of adjuvant used.

Stress and Oncogenesis

Only a few proto-oncogenes and activators and inhibitors of genes involved in cancerogenesis, stress response and toxic reactions were activated in the presence of antigen. In contrast, many more of these markers were upregulated after administration of the adjuvant alone. Since the spleen is located far from the administration site, the potential toxicological implications of this upregulation must be studied further. One example of special interest is the massive overexpression of cyclin D1 (26-fold as compared to N) in the ADJ sample. This gene has been identified as one of the key factors of cancerogenesis, since it is important for the 'restriction point control' of the cell-cycle.²⁴ In the presence of antigen, the gene for cyclin D1 is substantially downregulated and inhibitors for cyclin D1, such as p19ink4, p27kip1 and retinoblastoma-associated protein 1, are upregulated.²⁴ Again, the immune system seems to be alerted if apoptosis in response to harmful signals occur; and if harmful signals such as cancer markers appear or are upregulated. This hypothesis is supported by studies in which tumor growth is reduced or eliminated by the immune system after vaccination.⁴⁰

Why do we need adjuvants to elicit a protective immune response? Janeway suggested two reasons: to ensure effective antigen uptake into macrophages and, to provide co-stimulatory activity.⁴¹ According to Matzinger's theory, co-stimulation arises when endogenous, mostly unknown, signals originate from stressed or injured cells, and this is the ultimate controlling system of the immune response.⁴⁰ At this time, heat shock proteins and the LPS receptor are known to be involved.⁴² The upregulation of CD14 gene in the presence of adjuvant that we have observed in our experiments supports this suggestion.

In summary, our results indicate that the aluminum phosphate adjuvant regulates relatively few genes that are involved in the immune response, but a larger number of genes associated with inflammation, apoptosis, stress and oncogenesis. We propose that these various responses are an important part of the endogenous danger signals in the innate immune response. The TT-antigen is apart from being necessary for an adequate adaptive immune response, also necessary to modulate the response to the adjuvant. It seems to protect against adjuvant-mediated side effects. These findings show for the first time a finely tuned inter-

play between an antigen and the aluminum phosphate adjuvant in regulating signals for danger and protection.

We hope that further studies of this interplay will facilitate the discovery and development of new and more effective vaccine adjuvants.

MATERIALS AND METHODS

Animal Experiments

Female Balb/c mice, 10 weeks old (Charles River, Sweden), were randomly separated into groups of five animals. The mice were given an i.m. dose of vaccine (SBL Vaccin AB, Solna, Sweden) containing 1 Lf (2–2.5 µg) tetanus toxoid in aluminum phosphate adjuvant (TT_{ADJ}) at pH 6.0 (1:1) or a dose of adjuvant alone (ADJ) in a dose corresponding to the dose given with the vaccine. TT_{ADJ} was administered i.m. in the left hind leg quadriceps. The same doses were administered 3 weeks later. Two weeks after the booster, blood was collected from all mice by tail artery puncture for measurement of the systemic antibody-response using ELISA. Spleens were isolated, pooled for each group 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 1640 medium (Gibco/Invitrogen, Copenhagen, Denmark) supplemented with 292 µg ml⁻¹ L-Gln, 50 µM 2-mercaptoethanol, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 10% heat-inactivated FCS (Gibco). Aliquots of the cells (1.25 × 10⁶ cells ml⁻¹) were plated into cell culture flasks and incubated for 4 or 24 h, respectively, at 37°C in 5% CO₂ at approximately 90% humidity with 1 Lf tetanus toxoid (TT) per spleen. After incubation, the supernatants were recovered and used for the cytokine assay. 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 Ambion's Totally RNA™ kit (Austin, TX, USA). DNA contamination was removed using DNase I (Promega, Madison, WI, USA). Atlas mouse cDNA expression arrays with 588 genes (Clontech, Palo Alto, CA, USA) were used according to the manufacturers guideline. The level of expression was quantified by phosphor-imaging (Molecular Dynamics, Sunnyvale, CA, USA) and the 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. Array analyses were performed twice to assure intra-sample reproducibility. 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 in at least one sample were included.⁷ The Spotfire Pro program (version 4.2) was used to visualize expression changes. Genes were sorted and clustered by the programs Excel, Gene cluster²¹ and Treeview.²²

Analysis of mRNA Expression by RT-PCR

Total RNA was extracted as described above. RT-PCR reactions were performed using the Qiagen (Valencia, CA, USA) one-step RT-PCR kit and 1 µg total RNA according to the manufacturers guidelines with an annealing temperature of 55°C and 45 cycles. The following primers were used: Pax-5 forward-GACATGGAGGAGTGAATCAGC, reverse-GCCAC TGATGGAGTATGAGGA; SATB1 forward-TCTAGGAAGAG GAAGGCTTGG, reverse-GCCAACGAATCTGGTTCATA; LFA-1 β forward-TAATCCTGAGTTCGACCAACG, reverse-GACATGCAGCAACTTGCATTA; cyclin D1 forward-GAATCT GCATCACCCTGAGAG, reverse-TATTTTCATCCCTACCGCT GTG; TRAIL forward-GCAGCTGCAGGACAATTACTC; reverse-TGGTTCTCACCTTGTCCTTTG.

Antibody and Cytokine Analysis

TT specific antibodies were measured by the toxin-binding inhibition assay.⁴³ Cytokine protein levels in supernatants from restimulated spleen cells were analyzed by capture enzyme-linked immunosorbent assay (ELISA), using specific antibody pairs and recombinant cytokine standards (Pharmingen, San Diego, CA, USA) and ELISA plates (Nunc, Roskilde, Denmark) according to the manufacturer's instructions.

ACKNOWLEDGEMENTS

We thank Martha Granström, Karolinska Hospital, Stockholm, Sweden for the Ab determinations and SBL Vaccin, Solna, Sweden for the generous vaccine gift. This work was supported by the Swedish Board for Technical Development Grant p11381-1 and the Swedish National Network for Drug Development Grant B 6 3368/98.

DUALITY OF INTEREST

None declared.

REFERENCES

- 1 Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000; **406**: 782–787.
- 2 Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a non-clonal system of recognition. *Cell* 1997; **91**: 295–298.
- 3 Edelman R. Vaccine adjuvants. *Rev Infect Dis* 1980; **2**: 370–383.
- 4 Ross JS, Smith NP, White IR. Role of aluminium sensitivity in delayed persistent immunisation reactions. *J Clin Pathol* 1991; **44**: 876–877.
- 5 Malakoff D. Public health. Aluminum is put on trial as a vaccine booster. *Science* 2000; **288**: 1323–1324.
- 6 Bussiere JL, McCormick GC, Green JD. In: Powell MF, Newman MJ (eds). *Vaccine Design: The Subunit and Adjuvant Approach*. Plenum Press: New York, 1995, pp 61–79.
- 7 Teague TK, Hildeman D, Kedl RM, Mitchell T, Rees W, Schaefer BC et al. Activation changes the spectrum but not the diversity of genes expressed by T cells. *Proc Natl Acad Sci USA* 1999; **96**: 12691–12696.
- 8 Gutgemann A, Golob M, Muller S, Buettner R, Bosserhoff AK. Isolation of invasion-associated cDNAs in melanoma. *Arch Dermatol Res* 2001; **293**: 283–290.

- 9 Bucy RP, Panoskaltis-Mortari A, Huang GQ, Li J, Karr L, Ross M *et al*. Heterogeneity of single cell cytokine gene expression in clonal T cell populations. *J Exp Med* 1994; **180**: 1251–1262.
- 10 Finn PW, Kara CJ, Douhan J 3rd, Van TT, Folsom V, Glimcher LH. Interferon gamma regulates binding of two nuclear protein complexes in a macrophage cell line. *Proc Natl Acad Sci USA* 1990; **87**: 914–918.
- 11 Marsters SA, Pennica D, Bach E, Schreiber RD, Ashkenazi A. Interferon gamma signals via a high-affinity multisubunit receptor complex that contains two types of polypeptide chain. *Proc Natl Acad Sci USA* 1995; **92**: 5401–5405.
- 12 Musso T, Johnston JA, Linnekin D, Varesio L, Rowe TK, O'Shea JJ *et al*. Regulation of JAK3 expression in human monocytes: phosphorylation in response to interleukins 2, 4, and 7. *J Exp Med* 1995; **181**: 1425–1431.
- 13 Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998; **392**: 565–568.
- 14 Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997; **89**: 587–596.
- 15 Yin Z, Zhang DH, Welte T, Bahtiyar G, Jung S, Liu L *et al*. Dominance of IL-12 over IL-4 in gamma delta T cell differentiation leads to default production of IFN-gamma: failure to down-regulate IL-12 receptor beta 2-chain expression. *J Immunol* 2000; **164**: 3056–3064.
- 16 Takatsu K. Interleukin 5 and B cell differentiation. *Cytokine Growth Factor Rev* 1998; **9**: 25–35.
- 17 Xu-Amano J, Kiyono H, Jackson RJ, Staats HF, Fujihashi K, Burrows PD *et al*. Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J Exp Med* 1993; **178**: 1309–1320.
- 18 Cheng AM, Negishi I, Anderson SJ, Chan AC, Bolen J, Loh DY *et al*. The Syk and ZAP-70 SH2-containing tyrosine kinases are implicated in pre-T cell receptor signaling. *Proc Natl Acad Sci USA* 1997; **94**: 9797–9801.
- 19 Carter LL, Zhang X, Dubey C, Rogers P, Tsui L, Swain SL. Regulation of T cell subsets from naive to memory. *J Immunother* 1998; **21**: 181–187.
- 20 Rolink AG, Schaniel C, Andersson J, Melchers F. Selection events operating at various stages in B cell development. *Curr Opin Immunol* 2001; **13**: 202–207.
- 21 Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitareewan S, Dmitrovsky E *et al*. Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci USA* 1999; **96**: 2907–2912.
- 22 Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998; **95**: 14863–14868.
- 23 Vito P, Lacana E, D'Adamio L. Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science* 1996; **271**: 521–525.
- 24 Sherr CJ. Cancer cell cycles. *Science* 1996; **274**: 1672–1677.
- 25 Li XC, Demirci G, Ferrari-Lacraz S, Groves C, Coyle A, Malek TR *et al*. IL-15 and IL-2: a matter of life and death for T cells in vivo. *Nat Med* 2001; **7**: 114–118.
- 26 Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, Allison JP. CTLA-4-Mediated inhibition of early events of T cell proliferation. *J Immunol* 1999; **162**: 5813–5820.
- 27 Marengere LE, Waterhouse P, Duncan GS, Mittrucker HW, Feng GS, Mak TW. Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. *Science* 1996; **272**: 1170–1173.
- 28 Meager A. Cytokine regulation of cellular adhesion molecule expression in inflammation. *Cytokine Growth Factor Rev* 1999; **10**: 27–39.
- 29 Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants—a balance between toxicity and adjuvanticity. *Vaccine* 1993; **11**: 293–306.
- 30 Heller RA, Schena M, Chai A, Shalon D, Bedilion T, Gilmore J *et al*. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. *Proc Natl Acad Sci USA* 1997; **94**: 2150–2155.
- 31 Bei Qing L, Chen M, Whisler RL. Sublethal levels of oxidative stress stimulate transcriptional activation of c-jun and suppress IL-2 promoter activation in Jurkat T cells. *J Immunol* 1996; **157**: 160–169.
- 32 Chatta GS, Spies AG, Chang S, Mize GJ, Linsley PS, Ledbetter JA *et al*. Differential regulation of proto-oncogenes c-jun and c-fos in T lymphocytes activated through CD28. *J Immunol* 1994; **153**: 5393–5401.
- 33 Su B, Jacinto E, Hibi M, Kallunki T, Karin M, Ben-Neriah Y. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 1994; **77**: 727–736.
- 34 Surh CD, Sprent J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* 1994; **372**: 100–103.
- 35 Crispe IN. Death and destruction of activated T lymphocytes. *Immunol Res* 1999; **19**: 143–157.
- 36 Suzuki I, Fink PJ. The dual functions of fas ligand in the regulation of peripheral CD8+ and CD4+ T cells. *Proc Natl Acad Sci USA* 2000; **97**: 1707–1712.
- 37 Williams O, Gil-Gomez G, Norton T, Kioussis D, Brady HJ. Activation of Cdk2 is a requirement for antigen-mediated thymic negative selection. *Eur J Immunol* 2000; **30**: 709–713.
- 38 Rebollo A, Dumoutier L, Renaud JC, Zaballo A, Ayllon V, Martinez AC. Bcl-3 expression promotes cell survival following interleukin-4 deprivation and is controlled by AP1 and AP1-like transcription factors. *Mol Cell Biol* 2000; **20**: 3407–3416.
- 39 Mitchell TC, Hildeman D, Kedl RM, Teague TK, Schaefer BC, White J *et al*. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol* 2001; **2**: 397–402.
- 40 Matzinger P. An innate sense of danger. *Semin Immunol* 1998; **10**: 399–415.
- 41 Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 1989; **54**: 1–13.
- 42 Gallucci S, Matzinger P. Danger signals: SOS to the immune system. *Curr Opin Immunol* 2001; **13**: 114–119.
- 43 Hendriksen CF, vd Gun JW, Nagel J, Kreeftenberg JG. The toxin binding inhibition test as a reliable in vitro alternative to the toxin neutralization test in mice for the estimation of tetanus antitoxin in human sera. *J Biol Stand* 1988; **16**: 287–297.