ELSEVIER

Molecular and Cellular Endocrinology 152 (1999) 85–89

MCE
Molecular and Cellular Endocrinology

DNA immunization is associated with increased activity of type I iodothyronine 5′-deiodinase in mouse liver

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Received 27 January 1999; received in revised form 22 February 1999; accepted 22 February 1999

Abstract

Inflammatory cytokines in vitro are believed to be involved in the regulation of type I iodothyronine 5′-deiodinase (5′-DI) activity. The present study was undertaken to investigate in vivo effects of DNA immunization of mice on the 5′-DI activity in the liver. A mammalian expression vector encoding the β-galactosidase (pCMV-βgal) was used for intradermal immunization. Furthermore, immunostimulatory CpG motifs, which induce the expression of IL-6, IL-12, IL-18, TNF-α/β and IFN-γ were coinjected as oligodeoxynucleotides. From our data we conclude that the activity of 5′-DI in mouse liver when compared to non-immunized animals (100%) was found to be significantly enhanced by DNA immunization 2 weeks (175.7%) or 3 weeks (192.6%) after the plasmid injection. In addition, the activity of the 5′-DI in mouse liver was markedly enhanced 2 weeks (252.4%) or 3 weeks (243.3%) after the injection when CpG motifs were applied together with the plasmid DNA.

Keywords: DNA immunization; Type I iodothyronine 5′-deiodinase; Th-1 type response; IFN-γ; IL-4; Balb/c.

1. Introduction

Type I, iodothyronine 5′-deiodinase (5′-DI), an enzyme consisting of two identical 27-kDa subunits with one atom of selenium per molecule, catalyzes the conversion of the main secretory product of the thyroid, the prohormone thyroxine (T_4) to the active 3,5,3′-triiodothyronine (T_3), predominantly in liver, kidney, and thyroid (Köhrle et al., 1995). In liver, it is localized in the cytosolically oriented leaflet of the rough and smooth endoplasmic reticulum (Auf dem Brinke et al., 1979). The role of the cytokines, TNF, interleukins and interferones on the 5′-DI has been studied, but the results were contradictory depending on the experimental design and animal model used in the study (Köhrle, 1994). Recently, the study of the direct effects of cytokines upon the 5′-DI activity in phi1 rat liver cells has shown that three cytokines, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) stimulated the 5′-DI activity. These data do not support the hypothesis that inflammatory cytokines may mediate the euthyroid sick syndrome by causing inhibition of 5′-DI activity (Davies et al., 1997).

The aim of this study was to investigate the effects of DNA-based immunization (Donnelly et al., 1997) on the activity of 5′-DI in mouse liver. In contrast to the immunization with proteins, which are taken up by antigen presenting cells (APCs) and consequently are presented via MHC-II, plasmid DNA must first transfect cells to be translated into proteins. The predominant pathway, therefore, goes via MHC-I molecules, thus also leading to the activation of a cellular immune response. The major difference between the immune response elicited by conventional immunization with protein antigens and DNA is the induction of an infl-

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0303-7207/99/$ - see front matter
PII: S0303-7207(99)00054-4

ammatory Th-1 response type (Doe et al., 1996; Huang et al., 1996; Ulmer et al., 1996). Plasmid DNA contains unmethylated CpG dinucleotides flanked by two 5′ purines and two 3′ pyrimidines in the sequences. These—so called immunostimulatory sequences—were shown to provoke the innate immune system to produce a number of inflammatory cytokines (including IL-12, IL-18, IFN-α,β and TNF-α), which indirectly trigger the production of IFN-γ by T-cells and natural killer cells. Furthermore, CpG motifs were demonstrated to induce the expression of IL-6 in B-cells. These CpG motifs are 20-times as common in bacterial DNA than in mammalian DNA, and may serve as a 'danger signal' for the mammalian immune system (Krieg et al., 1995; Klinman et al., 1996; Sato et al., 1996; Kline et al., 1998; Hartl et al., 1999).

Considering the data concerning the in vitro effect of inflammatory cytokines on 5′-DI activity, DNA-based immunization offers the possibility to extend this aspect to a complex in vivo situation. For this purpose, we selected the molecule β-galactosidase (β-gal), which is known to be a strong inflammatory Th-1 type inducing immunogen in DNA-based immunization (Donnelly et al., 1997) because of the high number of CpG motifs within its coding sequence. Furthermore, in a second group of animals oligodeoxynucleotides containing CpG motifs were co-injected together with the plasmid DNA to enhance the induction of inflammatory cytokines of the innate immune system.

2. Materials and methods

2.1. Construction of DNA immunization vectors

The lac-Z (β-gal) cDNA was excised with Not I from pCMV-β (Clonetech, Palo Alto, CA) and cloned into the Not I site of the mammalian expression vector pCI (Promega Corporation) and designated pCI-βgal. The ability of the vector to express β-gal was confirmed by transfection of SSK-cells (a fibrosarcoma cell line) with plasmid followed by luminometric measurement of the β-gal activity in lysed cells after 48 h (Galacto-Light assay, Tropix, Bedford, MA, USA).

2.2. Oligodeoxynucleotide (ODN)

The ODN were purchased from MWG-Biotech GmbH (Ebersberg, Germany). The sequence of the CpG-ODN was 5′-GCTAGACGTTAGCGT-3′ (CpG motifs are underlined). The synthetic ODN was dissolved in LPS-free H_2O; the LPS content was < 1 ng LPS/mg DNA as determined by a limulus assay.

2.3. Immunization and experimental groups

Female BALB/c mice, aged between 5 and 6 weeks, were obtained from the animal breeding facilities in Himberg, Austria, and maintained at the central animal facility at the University of Salzburg according to the local guidelines for animal care. Sera were collected from all mice for antibody- and cytokine assays by tail bleeding and sodium azide was added to a final volume of 0.2%. The sera were stored at 4°C. Prior to injection, the DNA-vaccines were incubated at 95°C for 10 min and subsequently cooled at 4°C for 5 min. Groups of six mice were immunized intradermally into the shaved back (four spots each) with 100 μg of plasmid DNA in 100 μl sterile phosphate buffered saline, pH 7.2 (PBS) or with 100 μg plasmid DNA plus 50 μg of CpG-ODN in 100 μl PBS, respectively. Blood was taken at days 0 (preimmune serum), 14 and 21. Non-immunized animals and animals immunized with the vector lacking the insert served as control groups.

2.4. Determination of antigen-specific antibody production by ELISA

For ELISA, black 96-well high-bind immunoplates (FluoroNunc, Nunc, Roskilde, Denmark) were coated by overnight incubation at 4°C with purified recombinant β-gal protein at 1 μg/ml in PBS. Plates were washed with PBS-Tween using the AW1 automatic ELISA-plate washing device (Anthos Labtec, Salzburg, Austria) and blocked with PBS containing 3% BSA for 2 h at 37°C. Sera were added 1:100 in PBS to the left column of each plate and serial 1:3 dilutions in PBS were then made into subsequent columns. Preimmune sera served as negative controls. The plates were incubated for 1 h at 37°C and then washed. Horseradish peroxidase-conjugated detection antibody was added in PBS and incubated for 1 h at 37°C. Goat-anti mouse IgG (H + L, BioRad, Germany) and goat anti-mouse IgM (Pharmingen, CA) was used at 1:2000 dilution in PBS. Readings were referenced to commercial isotype standards. The luminometric assay was developed with Luminol (BM chemiluminescence substrate, Boehringer-Mannheim, Germany) diluted 1:1 in H_2O. Chemiluminescence (photon counts/s) was determined using a LucyI Elisa-plate Luminometer (Anthos Labtec, Salzburg, Austria). The obtained values were used to calculate the mean and standard error for each group of six mice.

2.5. Cytokine detection in supernatants of stimulated spleen cells

IL-4 and IFN-γ were both measured by ELISA employing the multiple antibody sandwich principle according to the protocol supplied by the manufacturer

(Genzyme, Cambridge, Massachusetts, USA). Briefly, 96-well microtiterplates (precoated with anti-mIFN-g or anti-mIL-4 antibody) were incubated with standard and test samples. Supernatants of cultured spleen cells were used undiluted whereas serum samples were diluted 1:10 in PBS. To determine the absolute protein-concentrations, standard calibration curves were obtained for each cytokine. After incubation with samples, a biotinylated anti-mIFN-γ or anti-mIL-4 antibody was added. Thereafter, plates were washed and horseradish peroxidase-conjugated streptavidin was added. After incubation, unbound material was removed by washing and a chemiluminescence substrate added to the plates. The signals were luminometrically evaluated according to the procedure described above. The photon counts measured were proportional to the concentration of IFN-γ and IL-4 present in the standards or samples. A standard curve was obtained by plotting the concentrations of cytokine standard versus the respective photon counts (the sensitivity was below 20 pg/ml). The cytokine concentrations were then determined using the standard curves.

2.6. Proliferation assay of isolated spleen cells

Spleens were minced in PBS and aggregated cells sedimented for 10 min. The supernatants containing a monodisperse cell suspension were washed three times in PBS and then resuspended in culture medium (DMEM) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml) and 1% heat-inactivated FCS. Cells (1×10^6/well) were cultured with recombinant β-gal (20 μg/ml) in 96-well, flat-bottom, tissue culture plates for 52 h at 37°C, 95% RH, 7.5% CO_2. Cells were pulsed with 25 μCi/ml [^{3}H]-thymidine (Amersham, Buckinghamshire, UK) for an additional 20 h and then harvested with a cell harvester (Skatron, Lier, Norway). [^{3}H]-thymidine incorporation was measured in a liquid scintillation counter (Beckman, Fullerton, USA).

2.7. Determination of the 5′-DI activity

The livers of BALB/c mice were homogenized by sonication (three times for 5 s) in ice-cold homogenization buffer containing 0.25 M sucrose, 20 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM D,L-dithiothreitol (DTT). 5′-DI activity was determined according to Leonard and Rosenberg (Leonard and Rosenberg, 1980) by the release of $^{125}I^-$ from [^{125}I]-3,3′,5′-triiodo-L-thyronine (reverse triiododo-L-thyronine, rT_3) using 2 μM nonradioactive rT_3 and 40 mM DTT in the absence (total iodothyronine deiodinase activity) or presence of 0.1 mM 6-*n*-propyl-2-thiouracil (PTU). The fraction of iodide release blocked by PTU was assigned to the 5′-DI activity. Specific activity of the 5′-DI was expressed as pmol of $^{125}I^-$ released per min and per mg of protein.

2.8. Estimation of protein

The protein concentration was determined by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as a standard.

2.9. Statistics

Data are expressed as mean ± SEM. Statistical significance was assessed using an unpaired Students *t*-test.

3. Results and discussion

Intradermal injection of 100 μg plasmid DNA encoding the β-gal induced the first onset of IgM antibodies after 14 days (Fig. 1a). At this time no IgG antibodies are detectable. The first peak of IgG antibodies appears 21 days after injection (Fig. 1b). This is in agreement with other experiments dealing with DNA immunization (Donnelly et al., 1997). The delayed onset of the humoral response after injection of plasmid DNA as compared to protein immunization (which is known to elicit antibody production within 1 week) can be explained by the fact that protein has to be first translated by the transfected cells in appropriate amounts to activate antigen-specific cells. As expected from the mechanisms involved in DNA-based immunization, mainly concerning the induction of cellular responses by antigen presentation, 2 weeks after injection of plasmid DNA, increased antigen-specific stimulation of spleen cells isolated from immunized animals can be detected (Fig. 2a).

The co-injection of immunostimulatory CpG motifs elicited a slight suppression of IgG antibodies. This effect is also in agreement with published data, which demonstrated a decrease of antibody titers after addition of CpG motifs (Lee and Sung, 1998). It can be explained by the ability of CpG motifs to enhance the IFN-γ production and thereby altering the intracellular processing of antigens via the proteasomal components LMP2 and LMP7 (Gacynska et al., 1993; Boes et al., 1994). On the other hand, coinjection of CpG motifs demonstrated a supporting effect with respect to antigen-specific proliferation, both 14 days as well as 21 days after injection.

However, these results only provide indirect indications for a running inflammatory Th-1 response induced by DNA-immunization. Therefore, the key cytokines for inflammatory Th-1 and non-inflammatory Th-2 responses, namely IFN-γ and IL-4, were measured in the supernatants of the proliferation cultures (Fig. 2). The results clearly point to a strong IFN-γ mediated Th-1 type response induced by DNA-immunization with β-gal. Three weeks after injection spleen cells from both groups produced significantly elevated IFN-γ titers

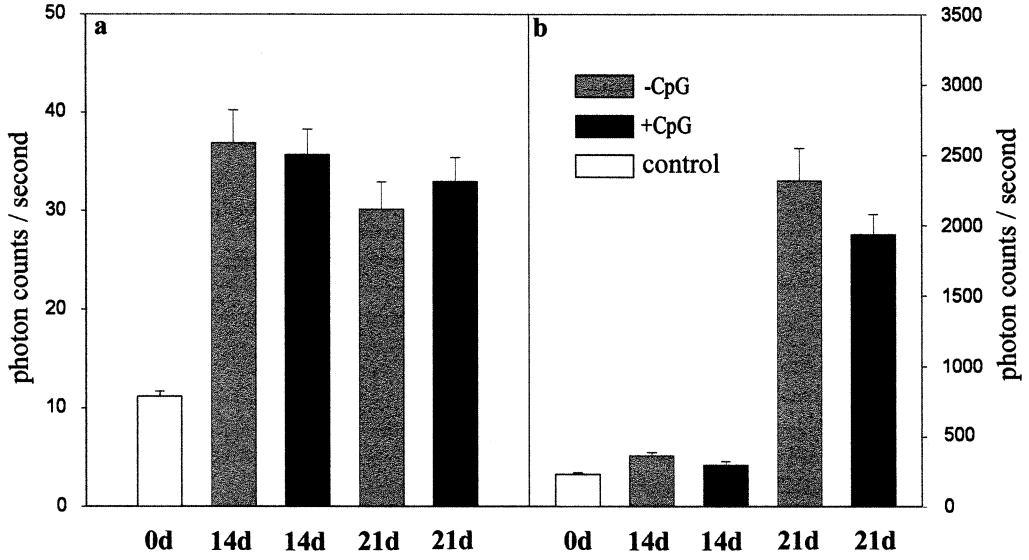

Fig. 1. ELISA of IgM (a, left) and IgG (b, right) immune responses after intradermal injection of 100 μg pCI-βgal (-CpG) and 100 μg pCI-βgal together with 50 μg CpG-ODN (+ CpG). Blood samples were taken on days 0 (control), 14 and 21 ($n = 6$ for each group). Recombinant β-gal served as antigen, serum dilution used for this graph was 1:100. Data are expressed as mean ± SEM.

(Fig. 2b), but no change of the IL-4 production could be detected (Fig. 2c). Application of CpG motifs elicited increased amounts of IFN-γ even 2 weeks after injection, and significantly enhanced the production after 3 weeks.

Considering these results we decided to measure the 5′-DI activity 14 and 21 days after injection of plasmid DNA. The first time point reflects the onset of the primary response with IgM production and antigen-specific proliferation, but only with a marginal increase of IFN-γ (without injection of additional CpG motifs). The second time point represents the mature primary response with IgG production, proliferative responses and significantly increased IFN-γ expression.

5′-DI appears to play an important role as 'gatekeeper' for the nuclear 3,5,3′-triiododo-L-thyronine receptors (Köhrle, 1994). As shown in Fig. 3, a marked ($P < 0.05$) enhancement in the 5′-DI activity in mouse liver was found in animals 2 and 3 weeks after DNA immunization with the plasmid encoding β-gal when compared to non-immunized mice. This activity in mouse liver was additionally increased 2 and 3 weeks after injection of plasmid DNA co-injected together with CpG motifs.

Our in vivo data seem to be in good agreement with those of Davies et al., 1997 who have shown the in vitro direct effect of inflammatory cytokines on the stimulation of the 5′-DI activity in phi1 rat liver cells. Further-

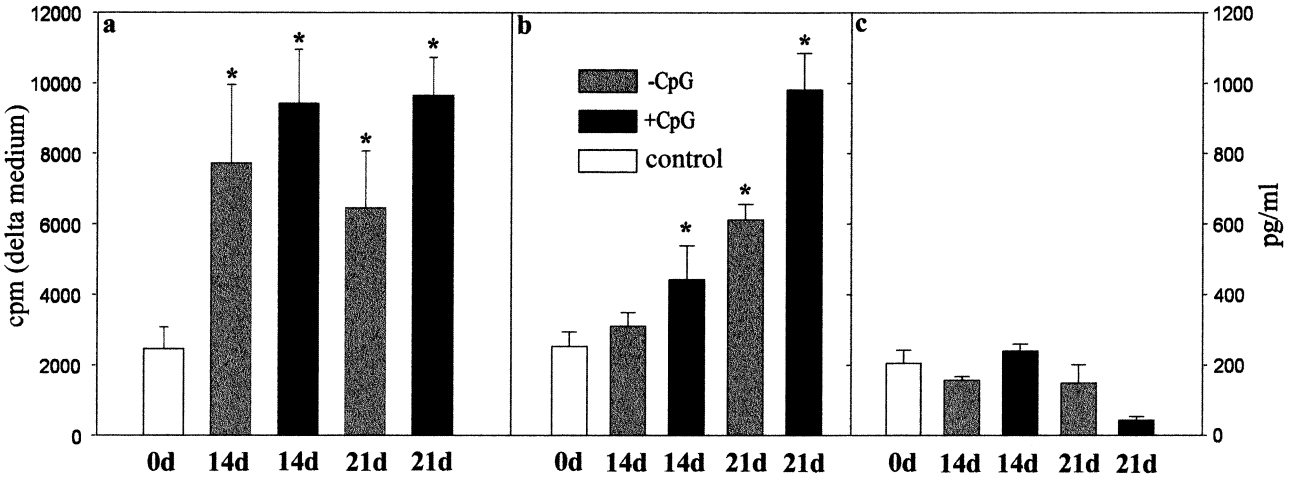

Fig. 2. Antigen-specific proliferation of isolated spleen cells (a, left) and ELISA of IFN-γ (b, middle) and IL-4 (c, right) in supernatants of proliferation cultures. Animals were immunized with 100 μg pCI-βgal (-CpG) and 100 μg pCI-βgal together with 50 μg CpG-ODN (+ CpG) and proliferation was performed on days 14 and 21 (control = values of non-immunized animals). Proliferation values are expressed in delta cpm (medium), mean ± SEM, cytokine values are expressed in pg/ml, mean ± SEM. *, $P < 0.05$.

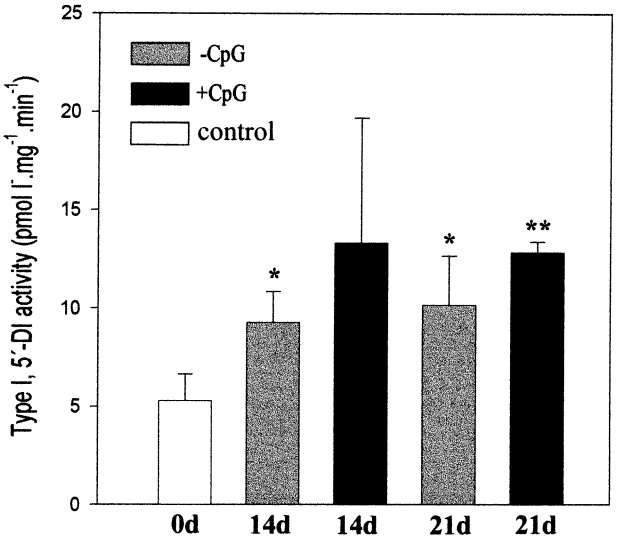

Fig. 3. The effect of DNA immunization with (+ CpG) or without application of CpG motifs (-CpG) on the activity of 5′-DI in the liver, 14 and 21 days after injection of plasmid DNA. The data are expressed as mean ± SEM. Control, non-immunized animals; *, $P < 0.05$; **, $P < 0.01$.

more, the correlation of the increase of IFN-γ with the enhanced 5′-DI activity by additional application of CpG motifs supports this interpretation.

CpG motifs present in the plasmid DNA (or given as a genetic adjuvant) are widely accepted as the major signal inducing the inflammatory Th-1 type response after DNA-based immunization (Donnelly et al., 1997; Hartl et al., 1999). However, it may be of interest for future studies to investigate the effect of a typical non-inflammatory Th-2 type immune response (induced by protein immunization) on the 5′-DI activity. Nevertheless, the present results might serve as an additional evidence not supporting the hypothesis that inflammatory cytokines inhibit the 5′-DI activity thus mediating the euthyroid sick syndrome.

Acknowledgements

This work has been supported in part by the VEGA Grant No. 2/6085/99 and by the Ludwig-Boltzmann Institute for Experimental Surgery (Professor O. Boeckl) and the Senat of the University of Salzburg.

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