RETINOIC ACID RECEPTOR STATUS IN MOUSE SPLEEN DURING A PRIMARY IMMUNE RESPONSE AGAINST β-GALACTOSIDASE

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Objective. Evaluation of the dynamics of all-trans retinoic acid receptor binding properties in mouse spleen nuclear extracts during a primary immune response against β-galactosidase.

Methods. Female BALB/c mice, aged between 5 and 6 weeks were immunized intradermally into the shaved back (4 spots each) with 100 μg β-galactosidase in 100 μl sterile phosphate buffered saline (pH 7.2) and blood was taken by tail bleeding on days 0 (preimmune serum), 4 and 6. Production of antibody in serum and the detection of cytokines (IL-4, IFN-gamma) from proliferation supernatants were determined by ELISA. Antigen-specific proliferation assay of isolated spleen cells was based on [^{3}H]-thymidine incorporation measured in a liquid scintillation counter. Both, the maximal binding capacity (B_{max}) and the afinity (K_a) of all-trans retinoic acid nuclear receptors (RAR) were evaluated according to Brtko (1994).

Results and conclusions. Injection of β-galactosidase induced the first detectable antibody responses on day 4 (IgM) and on day 6 (IgG). These points of time, reflecting the early and the mature immune response served to measure the antigen-specific proliferation and production of IL-4 and IFN-gamma in the supernatants of the proliferation cultures as well as all-trans retinoic acid receptor (RAR) binding characteristics in spleen nuclear proteins. The RAR B_{max} was significantly ($P<0.05$) decreased only at the time of the first specific IgG antibody production.

Conclusions. The data obtained indicate the involvement of RAR in the late phase of an in vivo immune response.

Key words: BALB/c mice – Protein immunization – β-galactosidase – Immune response – Nuclear retinoic acid receptor

Retinoids, in particular all-trans retinoic acid (RA), are essential for a normal development and homeostasis of vertebrates. RA action on the regulation of specific gene expression is mediated by three distinct nuclear retinoic acid receptor subtypes, retinoic acid receptor (RAR) α,β, gamma belonging to the steroid/thyroid/retinoid nuclear receptor superfamily. RARs are capable to function as transcriptional repressors in the absence of the specific ligand and potent activators upon binding of RA (Kliewer et al. 1992). The RA-RAR complexes regulate target gene expression through the binding to short cis-acting DNA sequences, hormone responsive elements (RAREs) near the promoters of target genes. Besides all-trans retinoic acid receptors, 9-cis retinoic acid receptors (RXRs) play an important role in the regulation of many intracellular receptor signalling pathways and can mediate ligand-dependent transcription, as well. The ability to recognize target genes is a particularly important aspect of nuclear receptor function. The RARs preferentially bind to the sequence AGGTCA on the DNA molecule. Specificity of the target gene recognition within the RAR subgroups is determined, at least in part by the orientation and spacing of two AGGTCA half-sites (Lazar 1993). Many of RAREs that consist of a direct repeat of two AGGTCA motifs separated by a five-base pair spacer have

been identified in a number of genes that respond to RA in vivo (Gudas 1994).

RA and RARs have been demonstrated to be involved in various aspects of the immune system. Antigen presenting cells (APC) like Langerhans cells upregulate their ability to present allo-antigens by RA (Meunier et al. 1994) and monocytes are induced to produce increased TGF-β and TNF-α levels (Szabo et al. 1994). Experiments using RAR gamma-transgenic animals indicated a role of RAR inducing cytotoxic T-lymphocytes in vivo and of skin graft rejection (Pohl et al. 1993). Expression studies in antigen-stimulated T lymphocyte cultures demonstrated an upregulation of RARα mRNA by RA thus pointing to a function of RARα as a ligand inducible transcriptional enhancer factor (Friedman et al. 1993). Retinoids act also on B cells, RAR ligands are able to inhibit cell activation and can prevent apoptosis in B lymphocytes (Lomo et al. 1998). Furthermore, retinoic acid induced gene transcription seems to have strong impacts also on the expression and regulation of adhesion molecules like ICAM-1 (Aoudjit et al. 1994, 1995; Bassi et al. 1995) and cytokines like interleukin-2 (De Grazia et al. 1994; Ballow et al. 1997), interleukin-4 (Racke et al. 1995) and IFN-γ (Cippitelli et al. 1996). Concerning the role of RA and RAR in the immune system, an increasing number of publications are focused on the regulation of apoptosis, i.e. RA can prevent activation-induced T cell apoptosis by inhibiting the induction of Fas ligand expression (Yang et al. 1994; Szondy et al. 1998).

In the present work we studied the dynamics of RAR binding properties in spleen cell nuclei, which are known to contain a high concentration of total RARs (Zhuang et al. 1995) during a primary immune response. Immunization was performed in a conventional way by a single intradermal injection of the protein β-galactosidase. The aim of the study was to answer whether RARs are involved in a primary immune response in the spleen cells of immunized animals.

Materials and Methods

Animals. Female BALB/c mice, aged between 5 and 6 weeks, were obtained from the animal breeding facilities in Himberg (Austria), and maintaned at the central animal facility at the University of Salzburg according to the local guidelines for animal care.

Immunization and experimental groups. Groups of eight mice were immunized intradermally into the shaved back (4 spots each) with 100 μg protein in 100 μl sterile phosphate buffered saline, pH 7.2 (PBS). Non-immunized animals served as control groups. Blood was taken on days 0 (preimmune serum), 4 and 6, the sera were collected for antibody assays and stored at –70 °C after addition of 0.2 % sodium azide.

Determination of 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-galactosidase 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/second) 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 eight mice.

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-γ or anti-mIL-4 antibody) were incubated with standard and test samples. Supernatants of cultured spleen cells were used undiluted. To determine the absolute protein-concentrations, standard calibration curves were obtained for each cytokine. After incubation with samples, a biotinylated anti-mIFN-gamma or anti-mIL-4 antibody was added. Thereafter, plates were washed and horseradish peroxidase-conjugated strepta-

vidin 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.

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 3 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 ($1x10^6$/well) were cultured with recombinant β-galactosidase (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 Coulter, Fullerton, USA).

Isolation of mouse spleen nuclei and extraction of retinoic acid receptors. Spleens of eight mice per each group were pooled at days 0 (control) 4 and 6 for the animals immunized with protein. The splenic tissue was minced and homogenized in 0.32 M sucrose, 1 mM $MgCl_2$, 0.1 mM PMSF and 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 1000 x g, the crude pellet washed and then mixed with 2.3 M sucrose containing 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and treated by isopycnic ultracentrifugation at 220 000 x g, 30 min. Nuclei were then washed twice in ice-cold SMCT buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.1 mM PMSF) once in the presence of 0.25 % Triton X-100 and once without. The retinoic acid receptors containing nuclear protein fraction was obtained directly from purified nuclei with a high ionic strength buffer containing 0.3 M KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, (pH 7.0) at 0 °C for 1 h and by subsequent ultracentrifugation at 135 000 x g (Torresani and De Groot 1975; Brtko 1994).

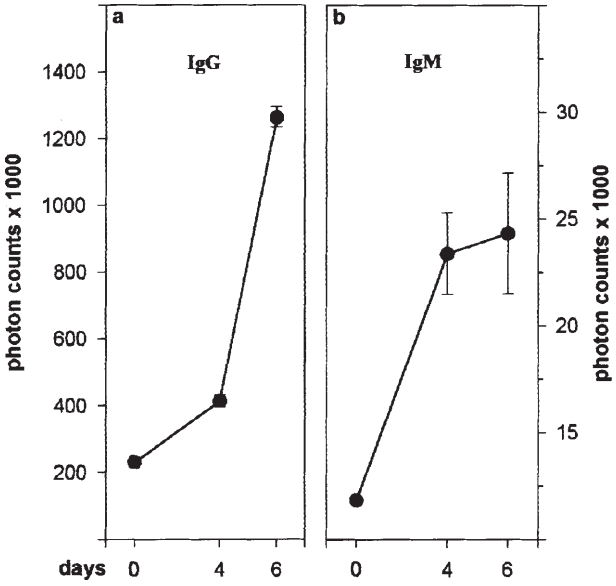

Fig. 1 ELISA of IgG (panel a) and IgM (panel b) immune responses after intradermal injection of 100 μg β-galactosidase (β-gal protein). Preimmune serum was taken at day 0. After a single injection blood was taken at days 4 and 6. Recombinant β-galactosidase served as antigen, serum dilution used for this graph was 1:100. Data (photon couints x 1000) are expressed as mean ± S.E.

Binding of labelled [11,12-^{3}H(N)] all-trans retinoic acid to nuclear receptors. The assays on labelled all-trans retinoic acid binding to nuclear proteins were performed at 20 °C in dark in a high ionic strength buffer (0.3 M KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl buffer, pH 7.0). Samples were incubated for 2 h with 0.66 nM of [11,12-^{3}H(N)] retinoic acid (spec. act. 1824.1 Gbq/mmol, NEN Research products; 3 μl ethanol solution per 0.5 ml sample) and increasing concentrations of all-trans retinoic acid (0.33 – 3.96 nM; 3 μl ethanol solution per 0.5 ml sample). Nonspecific binding of the labelled ligand which was determined by simultaneous incubation with 0.396 μM all-trans retinoic acid. After incubation, 0.5 ml of charcoal-dextran suspension (4.5 mg Norit A + 0.45 mg dextran/ml) in a high ionic strength buffer (pH 7.0) in dark at 0-4 ˚C was added to each sample. After short vortexing, the suspension was placed on an ice bath for 10 min, then vortexed and the supernatant was collected after 10 min centrifugation at 1500 x g. Then 0.5 ml of the supernatant was decanted, mixed with 5 ml INSTA-gel (Packard, U.S.A.) and its radio-

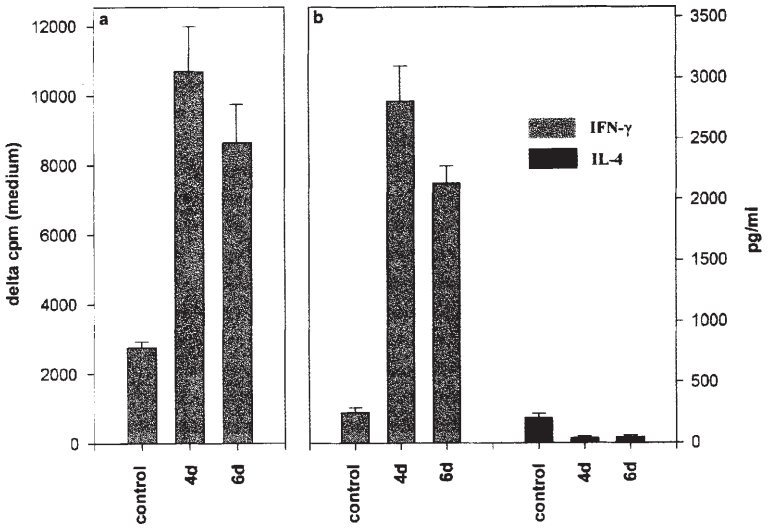

Fig. 2 Antigen-specific proliferation of spleen cells (panel a) and cytokines in supernatants of proliferation cultures (panel b). Both stimulation and cytokine measurement was performed 4 and 6 days after the injection. Stimulation values are expressed in delta/cpm (delta/medium), mean ± S.E.; cytokine data are in pg/ml as mean±S.E.

activity was quantified in a LKB model 1217 Rackbeta (Pharmacia, Sweden) liquid scintillation counter (Brtko 1994).

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

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

Results

Following the course of the humoral primary immune response against β-galactosidase by ELISA, the injection of protein elicited a strong IgM response after 4 days, whereas the production of IgG started at 6 days after protein immunization (Fig. 1). In order to determine the antigen-specific cellular immune reaction, spleen cells of immunized animals were stimulated with recombinant β-galactosidase, and [^{3}H] thymidine incorporation was measured (Fig. 2a). Immunization with protein induced proliferative responses 4 days as well as 6 days after injection. Additionally, the supernatants of the proliferation cultures were used to characterize the cytokine profiles concerning IFN-γ and IL-4. As shown in Fig. 2b, protein immunization caused the increase of the IFN-γ expression but not that of IL-4.

The characterization of the RAR (Fig. 3) revealed that 4 days after the injection of protein the concentration of nuclear receptors slightly increased (statistically not significant) and significantly decreased at the peak of responsiveness (P=0.0041) 6 days after the injection. No significant changes in the retinoic acid receptor affinity could be seen on day 4 or on day 6 after protein immunization.

Discussion

In our very recent study, we investigated in vivo effects of DNA-based immunization of mice on binding parameters of RARs in spleen nuclei, and the results clearly demonstrated that the concentration of RARs was significantly reduced in the late phase of the primary immune response, 21 days after injection of plasmid DNA encoding the gene for the model enzyme β-galactosidase (Brtko et al. 2000). This molecule from the bacterium Escherichia coli is known to be a strong immunogen (Sundaram et al. 1996; Davis et al. 1997) inducing both humoral and cellular immune responses after a single injection.

In the present work we have focused our attention on the conventional immunization method using the recombinant protein as a source of antigen. In contrast to the rather slow kinetics of the immune response

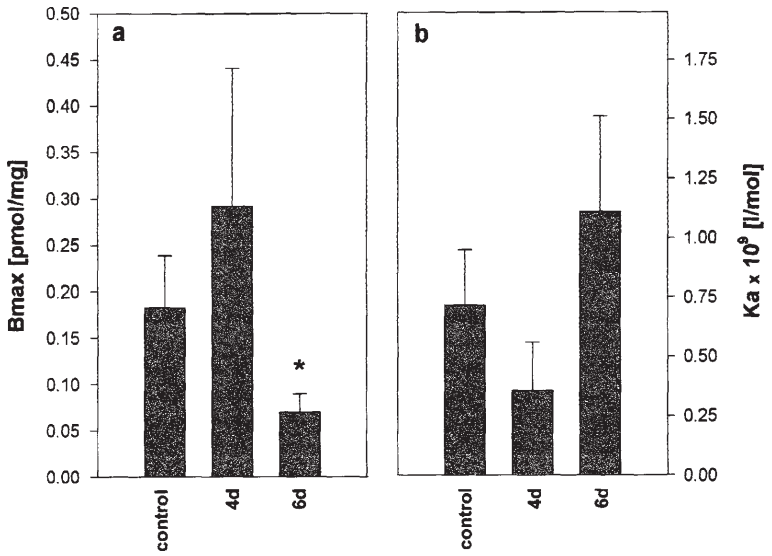

Fig. 3 Characterization of the concentration (B_{max}) and the affinity of the RAR (Ka) from spleen cells of animals immunized with recombinant β-galactosidase. B_{max} showed a tendency to increase at the first onset of IgM production and decreased significantly at the first onset of IgG production. Data are expresed as mean±S.E. * = P<0.05 compared to control values.

development after injection of DNA encoding the β-galactosidase, the injection of the protein itself induces a much faster response. The reasons therefore are that after conventional immunization the protein is immediately accessible for activation of antigen presenting cells (APCs) whereas after DNA immunization a series of events like transfection, transcription and in the end translation of protein must take place in order to start of the immune activation cascade. However, concerning the principle course of basic immune mechanisms both methods first induce the onset of IgM production followed by IgG antibody expression. In addition, like with DNA immunization, both antigen-specific proliferation as well as expression of IFN-γ was measurable first at the time of induction of IgM antibodies and with both methods no significant expression of IL-4 was detectable.

Since DNA immunization was found in vivo to down regulate nuclear all-trans retinoic acid receptors in mouse spleen cells in the late phase of the immune response as indicated by the specific IgG production (Brtko et al. 2000), our question was, whether the RAR specific binding characteristics in the spleens may exhibit a similar behaviour after conventional protein immunization. From both studies we can conclude that either protein immunization or DNA-based immunization elicited a significant diminution of RAR B_{max} at the first onset of IgG production.

At present, we cannot interpret the down-regulation of RARs by both the protein and the DNA immunization from a functional point of view since RARs are involved in various aspects of the immune system in a very complex context. However, the data clearly demonstrate the influence of in vivo immune responses on the RAR concentration in the spleen.

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