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Immunogenicity of autologous IgG bearing the inflammation-associated marker 3-nitrotyrosine

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Abstract

To explore the link between inflammation and autoimmunity, we analyzed the immunogenicity of 3-nitrotyrosine (NT)-bearing self-proteins, an inflammation-associated marker that is formed by nitration of protein tyrosine residues with peroxynitrite generated during inflammation. An interesting feature of NT is its structural similarity to a synthetic hapten, 4-hydroxy-3-nitrophenylacetyl (NP), with which some anti-DNA antibodies (Abs) have been reported to show cross-reactivity. We confirmed that some of anti-DNA monoclonal Abs (mAbs) obtained from MRL/lpr mice also bound NT as well as NP. Based on these findings, we examined whether NT-bearing autologous IgG (NT-IgG) as a model of NT-self proteins is immunogenic to induce a DNA-cross-reactive anti-NT Ab response in autologous normal mice. Anti-NT IgM and IgG Ab responses were elicited after the third immunization with NT-IgG, concomitant with an increase in anti-single stranded (ss)DNA titer. Interestingly, a part of anti-NT mAbs thus induced showed cross-reactivity with ssDNA, some of which used VH sequences that were highly homologous to those reported in anti-DNA Abs from NZB/WF1 mice. Splenic T cells primed with NT-IgG, but not with unmodified IgG, showed a proliferative response to the inducing antigen. Collectively, NT-IgG is immunogenic in autologous hosts, and can induce anti-NT Abs that are cross-reactive with ssDNA.

Keywords: Tyrosine-nitrated protein; Anti-DNA antibody; Immunological tolerance; Autoantibody

1. Introduction

What triggers the production of auto-Abs is a pivotal, but not a fully answered question in understanding the onset of a variety of autoimmune diseases. If autologous proteins are inducing Ags of autoimmune responses, how do they acquire immunogenicity? Generally, a majority of endogenous Ags is non-immunogenic due to immunological tolerance at T cell and/or B cell levels. However, it has been pointed out that autologous proteins may become immunogenic if they are structurally modified post-translationally under physiologic or pathologic conditions [1,2]. In inflamed tissues or apoptotic cells, a variety of chemically modified proteins have been shown to accumulate [1,3,4]. These chemical modifications include, for instance, transglutamination, deamidation, deimination, glycosylation, oxidation, nitration and proteolytic cleavage [1,4–7]. The consequence of these protein modifications may be generation or unmasking of new antigenic epitopes, which will stimulate relevant B cells and/or T cells, thus leading to the breakdown or bypass of tolerance.

Among protein modifications listed above, protein tyrosine nitration is widely recognized as a hallmark of inflammation that is associated with the up-regulation of inducible NO synthase [6]. Activated macrophages produce superoxide (O_2^-) and NO, which are converted to peroxynitrite (ONO_2^-), a powerful nitrating agent that forms 3-nitrotyrosine (NT) [5,8]. Tyrosine nitration is also catalyzed by a class of peroxidases utilizing nitrite and

Abbreviations: Ab, antibody; dsDNA, double stranded DNA; NP, 4-hydroxy-3-nitrophenyacetyl; NT, 3-nitrotyrosine; ssDNA, single stranded DNA

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hydrogen peroxide as substrates [9,10]. To explore the role of NT-bearing self-proteins in the induction of autoimmune responses, we investigated the immunogenicity of NT-bearing autologous IgG (NT-IgG) that was selected as a model. NT-IgG is considered to be formed in vivo under inflammatory conditions because NT-IgG has been reported to occur in synovial fluids of patients with rheumatoid arthritis [11]. NT may serve as a B cell epitope because both polyclonal and monoclonal anti-NT Abs have been prepared by immunization with NT-bearing heterologous proteins [12]. On the other hand, a T cell epitope on pigeon cytochrome *c* (PCC88–103) in which Y97 is nitrated has been shown effective in eliciting a cell-mediated immune response that is specific for NT-PCC88–103, but not for the native peptide [13]. To our knowledge, it has been, however, poorly investigated whether NT-bearing intact autologous proteins are able to induce T cell-dependent antibody (Ab) responses. An interesting feature of NT is the structural similarity to a synthetic hapten, 4-hydroxy-3-nitorphenylacetyl (NP). Anti-single stranded (ss) and double stranded (ds)DNA Abs from lupus mice are generally polyreactive [14,15], and have been shown to cross-react with NP-related haptens [16]. These prompted us to investigate following issues. (1) Do anti-DNA Abs from autoimmune mice show cross-reactivity with NT? (2) If so, NT-IgG-that may be formed endogenously is immunogenic to induce anti-NT Abs that are cross-reactive with DNA? Results suggest that NT-IgG is immunogenic in autologous hosts.

2. Materials and methods

2.1. Preparation of NT-antigens

Murine IgG was purified from pooled BALB/c mouse sera using a Protein G-column (Amersham Biosciences, Tokyo, Japan) according to the indication of the manufacturer. Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). Peroxynitrite in 0.1 M NaOH solution was purchased from Dojin Chemical Laboratory (Kumamoto, Japan). NT-bearing IgG was prepared by nitration of protein tyrosine residues using peroxynitrite as reported previously with some modifications [17,18]. Briefly, IgG was dissolved at 2 mg/ml in 0.05 M potassium phosphate buffer (pH 7.4) containing 25 mM potassium bicarbonate, and incubated at 37 °C. Peroxynitrite solution was added to the IgG solution at 1 mM under vigorous stirring, and kept at 37 °C for 1 min. This was repeated five times so that the final concentration of peroxynitrite became 5 mM, followed by dialysis against PBS. Nitration of BSA was done in a similar fashion. The number of NT residues on proteins were estimated by measuring the absorbance at 430 nm in 0.01 M NaOH solution with the molar extinction of 4400 M^{-1} cm^{-1}. $NT_{14–17}$-IgG and $NT_{8–10}$-BSA were prepared in this way. In the present experiments, NT_{14}-IgG and NT_8-BSA were used.

2.2. Mice and immunization

Male BALB/c, and male MRL/*lpr* mice were purchased from Japan Charles River Inc. (Kanagawa, Japan). BALB/c mice were immunized intraperitoneally with 100 μg of NT-IgG and 2 mg of alum. After 3 weeks, booster immunization with 50 μg of NT-IgG and 2 mg of alum was done for two to three times at a 2 weeks interval and bled 7 days after each immunization. Control mice were given alum alone or native IgG plus alum. All mice were treated in accordance with the guideline approved by The Committee of Laboratory Animal Care, Okayama University.

2.3. Measurement of Ab levels by ELISA

ELISA for anti-NT Abs was performed using microplates coated with NT-BSA. Each class of Abs bound to the plates was estimated with peroxidase-conjugated goat IgG Abs specific for mouse IgM (Southern Biotechnology Associates, Birmingham, AL) or mouse IgG (Vector Laboratories, Burlingame, CA) as described previously [19,20]. In all experiments presented here, we confirmed that anti-NT sera or mAbs did not show reactivity with unmodified BSA, and that all Abs bound to NT-BSA were released from the plate in the presence higher than 5×10^{-3} M of free NT, thus suggesting the specificity of the anti-NT Ab assay. Anti-ssDNA or ds-DNA titers were measured by using microplates coated with ssDNA or dsDNA prepared from calf thymus DNA as reported previously [21]. Calf thymus DNA (Sigma–Aldrich) was dissolved in SSC (0.15 M NaCl, 0.015 M Na citrate, pH 8), followed by purification with phenol extraction. dsDNA was obtained by treating the DNA with S1 nuclease. ss-DNA was prepared by boiling dsDNA for 10 min, and rapidly chilled in ice. Microplates were coated by 50 μg/ml of ssDNA or dsDNA, and blocked with 1% BSA.

2.4. Anti-NT/DNA mAb-secreting hybridomas

On day 5 after fourth immunization of BALB/c mice with NT-IgG, dissociated spleen cells were prepared and fused with a myeloma cell line, NSObcl-2 [22] that was given by Dr. B. Diamond (Albert Einstein College of Medicine, New York, NY). After limiting dilution, anti-NT mAb from each hybridoma was examined for the binding to ssDNA. Anti-DNA mAb-secreting hybridomas were generated from T cell-depleted spleen cells from MRL/*lpr* mice of 18 weeks of age in the same way.

2.5. Sequencing of VH genes used in anti-NT mAbs

Total RNA was isolated from each anti-NT mAb-secreting hybridoma with TRIZOL reagent (Life Technologies, Rockville, MD). cDNA was synthesized with Superscript II reverse transcriptase (Life Technologies). The VDJ region of the mAb was amplified by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA) and following primers: ARC-