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Research paper

Potentiation of antigen-specific antibody production by peptides derived from Ag85B of *Mycobacterium tuberculosis*

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ABSTRACT

To generate high-titer monoclonal antibodies, strong immuno-stimulation must be used for eliciting an intense cellular immune response. Here, we report that antigen-specific antibody production was potentiated by Peptide-25 derived from Ag85B of *Mycobacterium tuberculosis*, and that the production of antigen-specific IgG1 in particular was markedly potentiated; specifically, this occurred because the use of Peptide-25 resulted in an increase in the number of antigen-specific antibody-producing cells. We studied the activation of T cells by the peptide by examining gene expression. The observed expression pattern of GATA-3 and T-bet suggests that the peptide modulates the Th1/Th2 balance during immunization. This potentiation, which was remarkably high in BALB/c mice, could be applied in the immunization performed for monoclonal antibody production *in vivo* and *in vitro*.

1. Introduction

Antibodies are indispensable for research, diagnostics, and therapeutics. For effective use in these fields, antibodies that exhibit high affinity and specificity toward antigens are required. High-quality antibodies are also required against a wide range of targets, including antigens that exhibit low immunogenicity and antigens that are toxic. Furthermore, highly functional antibodies are required that can detect posttranslational modifications, distinguish between subtle differences present among members of protein families, modulate the activity of antigens, and distinguish between the structural differences of antigens. Obtaining high-quality antibodies against poorly immunogenic antigens and antibodies that exhibit the aforementioned functions is a challenging task.

To obtain high-titer antibodies, a strong immune response must be elicited after immunization. To stimulate the immune system, antigens are applied together with an adjuvant. The activity of an adjuvant results from the sustained release of the antigen and the stimulation of a local innate immune response that generates enhanced adaptive immunity. The strong cellular immune response and the long immune sustainability lead to the production of high-titer antibodies. For obtaining such antibodies, an adjuvant that is commonly used is Freund's complete adjuvant (FCA). FCA is a water-in-oil emulsion containing heat-killed mycobacteria, and its use is an effective means of potentiating cellular and humoral antibody response to injected antigens (Stills, 2005). Although FCA has been a mainstay in immunological research for decades, it exerts several undesirable side effects; for example, FCA occasionally elicits inflammation and is toxic to the host animal. Thus, alternatives to FCA must be considered, and to produce high-titer antibodies, adjuvants that activate the immune system

Abbreviations: FCA, Freund's complete adjuvant; CpG ODN, CpG oligodeoxynucleotide; MDP, Muramyl dipeptide; OVA, Ovalbumin; PBS, Phosphate-buffered saline; FBS, Fetal bovine serum; AP, Altered peptide.

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effectively and produce weaker side effects than FCA are required (Jackson and Fox, 1995). When generating antibodies, various immuno-stimulators and adjuvants have also been used previously, including other microorganism-derived compounds such as muramyl dipeptides (MDPs) and tripeptides, cytokines, aluminum compounds (*e.g.*, alum), polymeric microspheres (Gupta et al., 1998), nanoparticles (Malyala and Singh, 2010), and liposomes (Haensler, 2010); however, the stimulation by these reagents was not adequately strong, and thus, an optimal stimulator remains to be identified. The immuno-stimulatory nucleic acid CpG oligodeoxynucleotide (CpG ODN) has also been used as an immune-stimulator at the time of immunization (de Titta et al., 2013; Kato et al., 2011; Weeratna et al., 2000). CpG ODN is a short oligonucleotide that contains unmethylated cytosine–guanine dinucleotides that feature a specific base context. Exposure to CpG ODN results in extremely rapid and strong immune activation, and, when applied together with an antigen, CpG ODN produces high titers of antigen-specific antibodies.

Takatsu and Kariyone (2003) determined that Peptide-25 derived from Ag85B of *Mycobacterium tuberculosis* induced Th1 development. Peptide-25 (aa 240–254) of Ag85B (also known as α antigen and MPT59) is a major T-cell epitope. Peptide-25 is immunogenic in I-A^b mice and it induces the development of Th1 cells that express TCRVβ11Vα5. Peptide-25 was extensively studied as a Th1 inducer (Bold et al., 2011), and the immunization of C57BL/6 mice with ovalbumin (OVA) together with Peptide-25 was shown to lead to an enhancement of anti-OVA IgG2a production. The researchers concluded that Peptide-25 exhibits potent adjuvant activity in both the humoral- and cell-mediated immune responses that appear to be mediated by Th1 cells (Kikuchi et al., 2006).

Here, we report that Peptide-25 derived from Ag85B of *M. tuberculosis* elicits the potentiation of antigen-specific antibody production; specifically, the number of antigen-specific antibody-producing cells was increased when the peptide was applied together with antigens. This method could be applied in the immunization performed for monoclonal antibody production *in vivo* and *in vitro*.

2. Methods

2.1. Synthetic peptides

Synthetic peptides derived from Ag85B were prepared by Sigma-Aldrich Japan (Tokyo, Japan). These peptides were dissolved in distilled water or 100 mmol/L Tris–HCl (pH 7.4) to a final concentration of 1 mg/mL. The sequences of the synthesized peptide are shown in Table 1.

Table 1
Sequences of Peptide-25 and analogs.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Peptide-25	F	Q	D	A	Y	N	A	A	G	G	H	N	A	V	F
AP1	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–
AP2	–	–	–	–	–	–	–	V	G	A	A	–	–	–	–
AP3	–	–	–	–	–	–	–	V	H	A	A	–	–	–	–
AP4	–	R	G	I	–	–	–	–	–	–	–	–	–	–	–
AP5	–	–	–	–	–	–	–	–	–	–	–	–	–	E	I

2.2. Mice and immunizations

Approximately 6–8-week-old female BALB/c, C57BL/6, and C3H/HeN mice were obtained from SLC (Tokyo, Japan). The mice received intraperitoneal injections of 100 μg of keyhole limpet hemocyanin (KLH, Thermo Fisher Scientific, Waltham, MA) in FCA (Sigma-Aldrich, St. Louis, MO) or together with or without 10 μg of peptides in normal phosphate-buffered saline (PBS) in a volume of 0.2 mL. Several days after the immunization, 200 μL of blood was collected from the tail; serum was prepared from the blood and its titer against the antigen was measured using enzyme-linked immunosorbent assay (ELISA). Mice were sacrificed 30 days after immunization and their spleens were removed aseptically. The spleens were squeezed, and single-cell suspensions were prepared. The cells were washed once in RPMI-1640 (Sigma-Aldrich) and then resuspended in 10 mL of RPMI-1640 containing 10% fetal bovine serum (FBS). The red blood cells and the granule cells were removed using Lympholyte-M (Cedarlane Laboratories, Canada). All animals were cared for and maintained in accordance with the guidelines of the National Institute of Advanced Industrial Science and Technology.

2.3. ELISA

We coated 96-well ELISA plates with 50 μL of 5 μg/mL KLH per well. A blocking solution (Blocking Reagent for ELISA; Roche) was applied and the plates were incubated for 2 h. Subsequently, the plates were washed with PBS containing 0.05% Tween-20 (PBS-T), after which 50 μL of PBS containing the supernatant of stimulated splenocytes was added to each well. After the wells were washed, an alkaline phosphatase-labeled anti-mouse IgG (Chemicon, MA) was added. The amount of the antigen-specific antibody present was measured using an alkaline-phosphatase substrate kit (Sigma-Aldrich), and the plates were read using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA), at a wavelength of 405 nm. All experiments were conducted twice, and the average signal intensity was used in the analysis.

2.4. ELISPOT assay

The frequency of B cells producing antigen-specific IgGs was determined using the enzyme-linked immunospot (ELISPOT) assay. Multiscreen HA filtration plates (Millipore, Billerica, MA) were coated with KLH at a concentration of 10 μg/mL (50 μL/well) and incubated overnight at 4 °C. The plates were then blocked for 2 h at 37 °C with RPMI-1640 containing 10% FBS. After washing the plates with PBS, cells were added to the plates at a density of 5×10^5 cells/well. The cells were cultured for 24 h at 37 °C and in the presence of 5% CO_2. After the culture period, the plates were washed with PBS-T and incubated with diluted goat anti-mouse IgG conjugated with alkaline phosphatase (Chemicon) for 2 h at 37 °C. The plates were washed with PBS-T and then Sigma Fast BCIP/NBT solution (Sigma-Aldrich) was added and the plates were incubated at room temperature for 10 min. When the color development was complete, the number of spots was scored. To investigate the IgG subtypes, alkaline phosphatase-conjugated goat anti-IgG1, IgG2a, and IgG2b (Southern Biotech, Birmingham, AL) were used.

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