Journal of Immunological Methods 387 (2013) 308–311

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

A simple technique to enhance the humoral immune response to intracellular protein antigens in genetic immunizations

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ARTICLE INFO

Article history:
Received 3 July 2012
Received in revised form 29 August 2012
Accepted 29 August 2012
Available online 3 September 2012

Keywords:
DNA vaccine
Humoral response
Secreted
Intracellular
Antigens

ABSTRACT

A simple technique to enhance the humoral immune response to intracellular protein antigens in genetic immunizations is demonstrated in mice. In this approach, the intracellular protein is intentionally secreted from expressing cells as a chimeric protein, comprising an N-terminal secreted protein fused to the intracellular protein antigen. Using the *Leishmania chagasi* Ldccys1 cysteine protease (411CP) as an example of an intracellular protein antigen and both human and murine granulocyte colony stimulating factor (GMCSF) as examples of N-terminal secretion competent fusion partners in chimeric proteins, a humoral response in plasmid DNA immunized mice could only be detected by Western blotting when the expressed 411CP was secreted.

1. Introduction

Genetic immunization is an approach for inducing immune responses to protein antigens expressed in vivo. DNA vaccines are one method of genetic immunization that employs plasmid DNA as an immunization vector. Using this method, DNA expression plasmids containing a gene encoding a desired protein antigen are introduced in vivo where they are taken up into cells at the site of injection. Once inside the cells, the encoded antigen is expressed under the control of an appropriate promoter and an immune response to it may ensue. Numerous immunizations in humans and animals have been described using this approach (reviewed by Liu, 2010).

One challenging aspect of genetic immunization is obtaining an effective immune response in order to meet the desired outcome (e.g. antibody titers, cellular immune responses, protective immunity, etc.). Modifications to plasmid DNA composition and regulatory elements, co-expression of immunomodulators such as GMCSF, as well as a variety of formulation and mechanical delivery methods are some approaches used in order to improve immune responses (reviewed by Laddy and Weiner, 2006). Studies with DNA vaccines have shown that the humoral immune response against a target antigen, that is naturally secreted or membrane bound, is greater compared to that when secretion is prevented by removal of the secretory signal peptide coding (Boyle et al., 1997; Drew et al., 2000). Conversely, this concept could be extended to those proteins that would normally be expressed intracellularly using genetic immunization, where the humoral responses may be improved if it was possible to intentionally secrete such proteins from the expressing cells.

The simple attachment of a sequence encoding an N-terminal signal peptide to the 5′ ends of sequences encoding proteins that are normally not secreted has previously been shown to be insufficient for achieving secretion; instead, an effective technique to intentionally secrete proteins normally located intracellularly using a secretion module has been described (Farrell et al., 2000). The secretion module in an expression cassette encodes a full-length secreted protein with no stop codon, which is joined in-frame to the coding sequence

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http://dx.doi.org/10.1016/j.jim.2012.08.017

for an intracellular protein. The presence of the N-terminal secreted protein in the resulting expressed chimeric protein can "piggyback" the desired intracellular protein through the secretory pathway and clear of the expressing cell. We have coupled this technique with plasmid DNA immunization as a means to enhance the humoral immune response against a normally intracellular protein, *Leishmania chagasi* cysteine protease. *Leishmania* cysteine proteases are potential drug targets for the prevention of leishmaniases (Mundodi et al., 2002).

In this report, we describe the coupling of two different secretion modules [human granulocyte macrophage colony stimulating factor (hGMCSF) and murine GMCSF (mGMCSF)] to an intracellular protein antigen [*L. chagasi* Ldccys1 cysteine protease (411CP)] and evaluate the antibody response to genetic immunization of mice using Western blotting techniques. An antibody response against the intracellular antigen 411CP could only be detected when a secretion module was employed.

2. Materials and methods

2.1. Plasmid constructs

The mammalian expression vector for the hGMCSF-411CP fusion protein expression was generated in two steps. First, a 960 bp fragment containing the coding sequence for an *L. chagasi* cysteine protease (Ldccys1) cDNA clone coupled to an N-terminal hexahistidine–enteropeptidase tag was cut from the vector pIE1/153A.jhe(6hep) containing the 411CP open reading frame (ORF) (Mundodi et al., 2002) by a partial *Not*I/complete *Bam*HI digestion, and ligated into the vector pcDNA3.1 (Invitrogen) to yield pcDNA.411CP (vector capable of expressing the intracellular form of 411CP). In the second step, a 0.45 kb *Bam*HI fragment containing hGMCSF (with no stop codon) was cut out of the vector pIE1/153A.gmcsf(6hep) cat (Farrell et al., 2000) and ligated into the unique *Bam*HI site of pcDNA.411CP to yield pcDNA.hGMCSF-411CP (vector capable of expressing the hGMCSF-411CP fusion protein). For the construct expressing the mGMCSF-411CP fusion protein, two PCR primers, 5′-GGTGGGATCCATGTGGCTGCAGAATTTA CTTTTCCTG-3′ and 5′-GGTGGGATCCTTTTTGGA CTGGTTTTTT GCATTCAAAG-3′ were used to amplify a 0.44 kb product containing mGMCSF (with no stop codon) using a cDNA template from reverse transcribed mouse liver mRNA. Next, the vector pcDNA.hGMCSF-411CP was digested with *Bam*HI to remove the human GMCSF gene and the linearized vector was ligated with the 0.44 kb *Bam*HI-digested PCR fragment containing the murine GMCSF ORF to yield pcDNA.mGMCSF-411CP. All constructs were verified by DNA sequencing at the University of Calgary DNA Sequencing Facility (Calgary, Canada). Plasmid constructs were amplified in *Escherichia coli* and DNA was isolated using an endotoxin free maxi prep kit (Qiagen) for expression in CHO cells and immunization in BALB/c mice.

A schematic of the genes encoding the expressed proteins is shown in Fig. 1A. Expression of each construct was verified in vitro by transient expression in CHO cells and Western blotting as described previously (Farrell et al., 2000). Only GMCSF-containing fusion constructs were found to be secreted into the supernatant of CHO transfected cells (data not shown).

2.2. Immunizations

Six to eight week old female BALB/c mice were used for the plasmid immunizations. Groups of four mice were injected with different plasmid constructs. Fifty micrograms of endotoxin-free plasmid DNA was diluted in PBS in a total volume of 50 μL. For immunization, mice were injected into the hind quadricep on days 0, 14, and 21. Sera samples were collected from the ocular vein on day 28, clotted overnight at 4 °C to remove hemocytes, pooled and stored at −20 °C for later analysis. Pre-immune sera was also collected prior to immunization and confirmed to have no cross-reactivity to control samples by Western blotting.

2.3. 411CP protein samples

A fusion protein sample comprising insect juvenile hormone esterase (JHE) and 411CP (JHE-411CP, Fig. 1B) was used as a positive 411CP protein control for Western analysis, as described previously (Mundodi et al., 2002). Briefly, after subcloning the coding for JHE-411CP into the insect expression vector pIE1/153A (Douris et al., 2006), plasmid DNA was introduced into High Five insect cells (Invitrogen) using Lipofectin-mediated transfection (Life Technologies) and the culture supernatant containing the expressed JHE-411CP fusion protein was harvested after 60 h. The positive control supernatant samples were confirmed to contain the JHE-411CP fusion protein by Western analysis using both anti-JHE antiserum and anti-411CP antiserum (Mundodi et al., 2002). The negative control comprised cell culture supernatant from High Five cells transfected with control plasmid, pIE1/153A.

2.4. Western analysis

Analysis of sera samples was performed by Western blotting. Replicates of positive control (JHE-411CP fusion in 50 μL of insect culture supernatant) and negative control (50 μL of supernatant from insect cells transfected with control plasmid) protein samples and molecular mass markers were resolved by SDS-PAGE, and transferred by electroblotting to nitrocellulose membranes. Following transfer, the membranes were cut into strips which were blocked for 1 h at room temperature in phosphate-buffered saline-0.1% Tween-20 (PBST) containing 10% (w/v) skim milk powder (PBSTM). Each strip was then incubated overnight (at room temperature) with a small volume of PBST containing the various samples of mouse sera at a dilution of 1:100. The strips were washed twice for 15 min with PBST, and incubated for 2 h with PBSTM containing horseradish peroxidase-conjugated goat anti-mouse IgG. The strips were then washed twice with PBST, incubated with ECL chemiluminescent substrate (Amersham) according to the supplier's instructions, and exposed to an X-ray film.

3. Results

Western blots of membrane strips probed with various sera samples from sets of DNA immunized mice are shown in Fig. 1C.