Protein Deficiency Impairs DNA Vaccine-Induced Antigen-Specific T Cell but Not B Cell Response in C57BL/6 Mice

Tohru SAKAI1,2, Kaori MITSUYA1, Mari KOGISO1, Kaori ONO1, Tatsushi KOMATSU3 and Shigeru YAMAMOTO3

1Department of International Nutrition, Institution of Health Bioscience, The University of Tokushima
Graduate School, Kuramoto 3–18–15, Tokushima 770–8503, Japan
2Department of Clinical Nutrition, Osaka Prefecture University, Habikino 3–7–30, Osaka 583–8555, Japan
(Received May 1, 2006)

Summary DNA vaccination is a simple method to induce antigen (Ag)-specific immune response and has many potential advantages over other vaccines. Although people who need to receive vaccines often suffer undernutrition, there has been no study on how nutritional status affects the immune responses induced by DNA vaccination. The aim of this study was to determine the relationship between protein deficiency and DNA vaccine-induced immune responses. C57BL/6 mice were fed a 5% or 20% casein diet for 30 d. The mice were immunized with an ovalubumin (OVA)-expression plasmid by the gene gun-based method three times at 10-d intervals. Body weight and serum albumin concentration in protein-deficient mice were significantly lower than those in mice fed the 20% casein diet (p<0.01, p<0.05). The percentage of OVA-specific CD8+ T cells was significantly decreased in the 5% casein group compared to that in the 20% casein group (p<0.05). Furthermore, CD4+ T cells from mice fed the low-protein diet showed lower interleukin (IL)-2 production than did those from the 20% group. In contrast to the T-cell function, protein deficiency did not affect OVA-specific Ab responses (p>0.05). These results suggest that protein deficiency impairs the induction of Ag-specific T-cell but not B-cell response in DNA-immunized mice. Our observation indicates that, in addition to development of an effective DNA vaccine, the management of nutritional state is important for the prevention of infectious disease by DNA vaccination.

Key Words DNA vaccine, protein deficiency, tetramer, cytotoxic lymphocyte, antibody response

Undernutrition is a strong determinant of morbidity and mortality from infection, especially in young children. Globally, it is estimated that 10.4 million children under the age of five die each year. Infections are the underlying cause of death for the majority: acute respiratory infections, 24%; diarrhea, 19%; malaria, 7%; and measles, 6%. However, undernutrition is the main underlying or associated cause in 49% of all deaths (1). The severity of undernutrition determines the severity of infection and risk of death (2–7). Undernutrition and infection interact as a vicious negative cycle. A large number of animal studies have demonstrated adverse effects of a low-protein diet on immunity (8, 9), and these effects have been confirmed in a human setting involving protein-energy malnutrition (10–13). In severely undernourished children, there may be multiple foci of infection (14–16). An inadequate or low-protein diet diminishes the immune responses and increases susceptibility to infection because immune defenses are dependent on cell replication and the production of proteins with biological activities; for example, immunoglobulins, cytokines, and acute phase proteins.

Administration of plasmid DNA expression vectors has been shown to result in corresponding protein expression in vivo, generation of humoral and cell-mediated immune responses, and protective efficacy in animal models of various infectious diseases (17, 18). DNA vaccines can induce protective immunity without the need for live organisms, replicating vectors or harmful adjuvants, and may therefore offer significant advantages in terms of practicality and safety over certain other approaches. Furthermore, DNA vaccination is simple and inexpensive, and DNA vaccines can be heat-stable. These properties are essential for vaccination in developing countries. Recently, there has been significant progress in DNA vaccine development targeting infections and tumors. In 1998, the first human clinical trial of malaria DNA vaccination targeting Plasmodium circumsporozoite protein (CSP) was carried out (19). DNA vaccinations have since been given to human subjects for prostate cancer (20), melanoma (21), HIV (22) and HBV (23).

DNA vaccination is an expectant new vaccine strategy. However, it is not clear how the nutritional state affects antigen (Ag)-specific immune induction by DNA vaccination. This point is crucial because people in developing countries and hospitalized patients in west-
ern countries who require vaccines often suffer severe undernutrition. In this study, we investigated the relationship between protein deficiency and DNA vaccine-induced immune response in a mouse model.

**MATERIALS AND METHODS**

*Mice.* Female C57BL/6 mice were purchased from SLC (Shizuoka, Japan). Mice were housed at the Animal Center of Tokushima University under specific-pathogen-free conditions. The room was maintained on a 12-h light:dark cycle at 24°C and 40–50% relative humidity.

This study conformed to the guidelines for the care and use of laboratory animals of the University of Tokushima Graduate School Institution of Health Science.

**Experimental diets.** Mice were pre-fed normal chow for 1 wk, after which they were separated into two groups and fed experimental diet (Table 1) for 30 d. Diets were replaced and food intake was measured every 2 d. Body weight was measured once a week. Diets were replaced and food intake was measured every 2 d. Body weight was measured once a week.

**Serum albumin concentration.** Serum albumin concentration was determined by the Albumin B test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) based on the bromcresol green method.

**Plasmid and genetic immunization.** Ovalbumin (OVA) expression plasmid, pCI-OVA, was kindly provided by Dr. T. Nagata (Hamamatsu University School of Medicine, Shizuoka, Japan). A pCI-OVA expresses OVA as an intact form (24). Plasmids were grown in *Escherichia coli* DH5α, prepared using a Qiagen Plasmid Maxi kit (Qiagen, Chatsworth, CA, USA) and purified using a Qiagen Plasmid Maxi kit (Qiagen, Chatsworth, CA, USA). The purified pCI-OVA was precipitated onto gold particles of 1.6 μm in diameter as previously reported (25). Briefly, 12.5 mg of gold particles were suspended in 200 μL of 0.1 M sperumidine, mixed with 200 μL of 20 μg of plasmid, and then mixed with 200 μL of 0.05 M CaCl₂. The plasmid/gold particles were then suspended in 6 mL of ethanol containing polyvinylpyrrolidone at 0.05 mg/mL. Next, the particles were coated on the inner surface of a tube using a tube loader, and the tube was cut into 0.5-inch segments to result in the delivery of 0.125 mg gold particles and 2 μg plasmid DNA per in vivo transfection. Plasmid DNA was delivered to the shaved abdominal skin of mice in three shots using a Helios Gene Gun (Bio Rad, NY, USA) at a helium pressure of 300 psi. Immunization was performed three times at 10-d intervals.

**Tetramer staining.** Ten days after the final immunization, splenocytes from nonimmunized and immunized mice were harvested. Splenocytes were stained with fluoresceinisothiocyanate (FITC)-conjugated antirat CD8 mAb and phycoerythrin (PE)-conjugated MHC class I-SIINFEKL tetramer (ProImmum Ltd., Oxford, UK) for 30 min at 4°C in 50 μL of FACS buffer (PBS containing 0.5% BSA and 0.02% of sodium azide). The cells were then washed twice with FACS buffer and analyzed on a FACS calibur using CellQuest software (Beckton Dickinson, Mountain View, CA, USA) after gating out dead cells using forward and side light-scattering.

**Interleukin (IL)-2 production.** CD4⁺ T cells were purified from splenocytes using a CD4⁺ T-cell isolation kit (Miltenyi Biotec GmbH, Germany) according to the manufacturer’s instructions. Purified CD4⁺ T cells (5 × 10⁶ cells/mL) were co-cultured with 200 μg/mL of OVA in the presence of mitomycin C-treated C57BL/6 mouse spleen cells (5 × 10⁶ cells/mL) or with plate-bound anti-CD3 mAb (10 μg/mL) (eBioscience, CA, USA) in complete RPMI 1640 medium (supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM 2-mercaptoethanol) for 72 h. After culturing, supernatants were collected and the concentration of IL-2 was determined using an IL-2 ELISA kit (eBioscience).

**OVA-specific Ab response.** Serum OVA-specific Ab levels were measured by ELISA using microplates coated with 10 μg/mL OVA. Serially diluted serum was added to the wells, incubated at room temperature for 2 h, and then washed with PBS containing 0.05% Tween 20. Alkaline phosphatase (AP)-conjugated antimouse IgG, IgG2a, IgG2b, or IgG1 (Southern Biotechnology Associates Inc., AL, USA) was diluted 1,000-fold, added to the wells, and then incubated for 2 h at 37°C. After washing, enzymatic activity was visualized using a substrate, p-nitrophosphate. OD was measured using a test wavelength of 415 nm.

**Statistical analysis.** The statistical significance of data was determined by Student’s *t*-test. Data are expressed as means ± SD. Significance of difference was accepted at *p* < 0.05.

---

**Table 1. Composition of experimental diet.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>20% casein</th>
<th>5% casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200¹</td>
<td>50</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>447</td>
<td>546</td>
</tr>
<tr>
<td>Sucrose</td>
<td>223</td>
<td>273</td>
</tr>
<tr>
<td>Soy bean oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin mixture²</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture³</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cellulose</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

¹g/kg diet.
²Vitamin mixture was composed of (mg or IU/kg diet): retinal acetate, 5,000 IU; ergocalciferol, 1,000 IU; tocopherol acetate, 50 mg; manadion, 52 mg; thiamine chloride, 12 mg; riboflavin, 40 mg; pyridoxine-HCl, 8 mg; vitamin B12, 0.005 mg; ascorbic acid, 300 mg; d-biotin, 0.2 mg; folic acid, 2 mg; calcium pantothenate, 50 mg; p-aminobenzoic acid, 50 mg; niacin, 60 mg; inositol, 60 mg; choline chloride, 2,000 mg.
³Mineral mixture was composed of (mg/kg diet): CaHPO₄·2H₂O, 7,280; KH₂PO₄, 12,860; NaH₂PO₄, 4,680; NaCl, 2,330; Ca-lactate, 17,750; Fe-citrate, 1,590; MgSO₄, 3,590; ZnCO₃, 55; MnSO₄·4–5H₂O, 60; CuSO₄·5H₂O, 15; KI, 5.
RESULTS

Body and organ weights, serum albumin

Although total food intake in the experimental groups did not differ, the body weight of mice fed the 20% casein diet significantly increased while that of mice fed 5% casein did not during the experimental periods. In the final experimental period, gain of body weight in mice fed the 5% casein diet was significantly less than that of mice fed the 20% casein diet. To evaluate the effect of protein deficiency, we determined the level of serum albumin and spleen weight. Protein-deficient mice showed lower serum albumin levels and spleen weights than did mice fed the 20% casein diet (Table 2). We further examined lymphocyte subsets, including CD4, CD8, B220 and DX5 Ag-positive cells in the spleen, but a significant difference was not found in any populations (data not shown).

OVA-specific T-cell responses

One of the advantages of DNA vaccination is that it can induce an Ag-specific CD8 response, which is crucial for protection against infectious diseases and tumors. First, to evaluate the Ag-specific CD8+ T cell induction in protein-deficient mice, we determined the proportion of OVA-specific CD8+ T-cells by spleen cells stained with SIINFEKL/H-2Kb tetramer. Figure 1 shows that SIINFEKL/H-2Kb tetramer enables the detection of OVA-specific CD8+ T cells and quantification of the proportion of these cells in OVA DNA-immunized mice. Using this method, we compared the proportions of OVA-specific CD8+ T cells in the 5% and 20% casein groups. In the 20% casein group, DNA immunization with OVA DNA induced a significantly large amount of Ag-specific CD8+ T cells, 2.8 ± 1.5% of CD8+ T cells. The proportion of OVA-specific CD8+ T cells in the 5% casein group was 0.7 ± 0.2%, significantly less than that in the 20% casein group (p < 0.05) (Fig. 2). Next, we purified CD4+ T cells from spleen cells and stimulated them with OVA or anti-CD3 mAb in vitro to determine IL-2 production. IL-2 production in the 5% casein group was less than that in the 20% casein group for both OVA stimulation and anti-CD3 mAb stimulation (Fig. 3).

OVA-specific B-cell responses

Finally, OVA-specific Ab response was estimated by ELISA. In contrast to T-cell response, the low-protein diet did not affect humoral immune response. As shown in Fig. 4, OVA-specific IgG, IgG1 and IgG2a levels in mice fed the 5% casein diet and the levels in mice fed the 20% casein diet were comparable. With regard to the IgG2b subclass, the level in mice fed the 5% casein diet showed a tendency to decrease, but the levels in mice fed the 5% casein diet and mice fed the 20% casein diet were not significantly different.

---

Table 2. Weight gain, spleen weight and serum albumin.

<table>
<thead>
<tr>
<th>Groups</th>
<th>20% casein</th>
<th>5% casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (g)</td>
<td>2.5 ± 0.91</td>
<td>0.0 ± 1.12</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>79.8 ± 10.8</td>
<td>64.8 ± 7.44</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>3.8 ± 0.4</td>
<td>3.4 ± 0.22</td>
</tr>
</tbody>
</table>

1 Values are means ± SD for seven mice.
2 Significantly different from the 20% casein group, p < 0.01.
3 Significantly different from the 20% casein group, p < 0.05.

---

Fig. 1. Flow cytometric analysis of SIINFEKL/H-2Kb tetramer-specific CD8+ T cells. The histogram shown is representative data for the detection of OVA-specific CD8+ T cells. Spleen cells from OVA expression plasmid-immunized (left panel) and unimmunized mice (right panel) were stained with FITC-conjugated anti-CD8 mAb and with PE-conjugated SIINFEKL/H-2Kb tetramer as described in “Materials and Methods.” Data were collected for 200,000 events, gating on live cells only. The number shown represents the percentage of these cells within the total cell population.
Fig. 2. Percentage of OVA-specific CD8+ T cells in mice immunized with OVA DNA. Spleen cells from mice fed 5% and 20% casein diets were stained with FITC-conjugated anti-CD8 mAb and with PE-conjugated SIINFEKL/H-2Kb tetramer. OVA-specific CD8+ T cells were defined as both CD8- and tetramer-positive cells. The percentage of OVA-specific CD8+ T cells was calculated as follows: (percentage of CD8 and tetramer-double positive cells/percentage of CD8-positive cells) x 100. Data are expressed as means±SD for seven mice. Results are representative of two independent experiments.

Fig. 3. OVA-specific IL-2 production from CD4+ T cells in mice immunized with OVA DNA. Spleen cells from seven mice were pooled and a single cell suspension was prepared from those. CD4+ T cells were purified as described in “Materials and Methods” and co-cultured with OVA or anti-CD3 mAb in vitro for 72 h. Production of IL-2 was determined by ELISA. Each value is the mean±SD for four well observations. Results are representative of two independent experiments.

Fig. 4. OVA-specific Ab responses in mice immunized with OVA DNA. Serum was collected from DNA-immunized mice 10 d after final DNA immunization. Levels of OVA-specific IgG, IgG1, IgG2a, and IgG2b serially diluted serum were quantified by ELISA. Data are expressed as means±SD for seven mice. Results are representative of two independent experiments.
DISCUSSION

In the present study, we evaluated how protein deficiency affects Ag-specific immune responses induced by DNA vaccination. It has been shown that protein-energy malnutrition affects various aspects of immune function in animal models and humans. However, with regard to vaccine-induced immunity, studies in which the effect of protein deficiency was investigated have been limited. The influence of dietary protein on the protective effect has been investigated using Bacille Calmette-Guerin (BCG) vaccine in a guinea pig model. In that study, protein deficiency resulted in diminished tuberculin reactions and loss of BCG-induced protection against virulent Mycobacterium tuberculosis challenge (26). Other studies have suggested that reduced T-cell number and their function are responsible for the impairment of protective immunity against M. tuberculosis (27–29). In human subjects, most of the findings regarding vaccine-induced immunity and nutritional state are controversial (12, 30, 31), and these have not been addressed using a new vaccine method. In this study, we focused on DNA vaccination and carried out experiments using an animal model since a DNA vaccine has recently been developed and used in human clinical trials.

Protein deficiency has been shown to be impaired by Ag-induced T- and B-cell responses in animal models (26–29, 32). DNA vaccination can induce both T- and B-cell responses as well as Ag immunization. The generation of Ag-specific CD8+ T cell is a characteristic property of DNA vaccination, while an induction of Ag-specific CD8+ T cell is not observed in Ag immunization (18). We mainly discuss, in this section, the effects of protein deficiency on the generation of CTL response and humoral immunity induced by DNA vaccination.

Cytotoxic T-cell response is crucial for protection against intracellular-infected pathogens and tumors. In a rodent malaria model, a DNA vaccination with CSP gene protected against infection, but this protection was abolished by treatment with anti-CD8 mAb in vivo (33). This suggests that Ag-specific CD8+ T cells play a role in protection. The contribution of CD8+ T cells to protective immunity has also been reported for intracellular pathogens (34), HB virus (35) and tumor (36). We found, in the present study, that protein deficiency impairs the induction of Ag-specific CD8+ T cells as estimated by tetramer staining (Fig. 2). A study on protein deficiency and immune function has been carried out, and their relationship has been shown (37). However, little is known about cytotoxic lymphocyte (CTL) generation in a state of protein deficiency because exogenous Ag cannot induce CTL responses (18). Therefore, an important finding in this study is that protein deficiency significantly reduces the frequency of Ag-specific CD8+ T-cell induction, which may lead to a reduction in protective immunity. Some mechanisms responsible for reduced CTL induction have been speculated. T cells play an important role in cell-mediated immunity. Ag-specific T cells are generated by the recognition of an antigenic peptide presented on MHC molecules of Ag-presenting cells through the T-cell receptor (TCR). Zhang et al. examined the Ag presentation ability of dendritic cells in protein-deficient weaning mice and showed their ability is sustained despite advanced weight loss (38). In a starvation condition, a decrease in the total number of dendritic cells and Ag-presenting ability has been reported (39). In our study, the contribution of Ag-presenting cells to the reduction of CTL generation is not clear. Other than dendritic cells, CD4+ T cells are thought to contribute to CD8+ T-cell generation. After exposure to Ag, CD8+ T-cell responses proceed through an ordered sequence of developmental events (40). The role of helper T (Th) cells in initiation and promotion of the development of CD8+ T cells in vivo is controversial, but results of recent studies indicate that CD4+ T cells are required for secondary expansion and memory CD8+ T cells (41, 42). We examined Ag-specific CD4+ T-cell response by measuring IL-2 production. OVA-specific CD4+ T-cell response was decreased in mice fed a 5% casein diet compared to that in mice fed a 20% casein diet (Fig. 3). In addition, reduced CD4+ T-cell response was also observed when stimulated with anti-CD3 mAb. These observations suggest that impaired Ag-specific CD4+ T-cell response might be responsible for reduced Ag-specific CD8+ T-cell generation.

In many animal studies, protein deficiency led to a decrease in humoral response. Decreased Ab response and delayed-type hypersensitivity (DTH) against keyhole limpet hemocyanin (KLH) was reported in KLH-immunized rats (32). In contrast, protein deficiency has been shown to marginally affect humoral immunity in human subjects. A difference was not found between the responsiveness to a recombinant HB vaccine in infants with protein-calorie malnutrition and that in healthy infants (31). Furthermore, Ag-specific Ab levels following tetanus toxoid immunization are unaffected in malnourished children (30). In our study, OVA-specific Ab response in mice fed 5% casein was not decreased compared to that in mice fed 20% casein (Fig. 4). OVA is known to be a thymus-dependent Ag. Although analysis of CD4+ T-cell function revealed that the function of Th cells in protein-deficient mice is impaired (Fig. 3), the level of Ab production was unaffected (Fig. 4). This fact shows that humoral immune response is less sensitive to protein deficiency than is cellular immunity, as reported in human subjects. In addition, we used a gene gun for in vivo gene transfer in this study. Gene gun methods have been shown to preferentially induce Th2 immune response (43), which is crucial for humoral immune response. Therefore, one of the mechanisms for sustained Ab production might be related to dominant induction of Th2 rather than Th1 response by gene gun-based immunization.

DNA vaccination is expected to enable the prevention of infectious diseases and tumors. Many techniques have been used in an attempt to develop an effective DNA vaccine. Fusion of L-selectin (44), HSP70 (45) or ubiquitin (46) DNA to target Ag DNA, and co-adminis-
tation with cytokine DNA (47) or Flt-3 ligand DNA (48) have been shown to enable the modulation of Ag-specific immune response, resulting in the enhancement of protective immunity. Much interest has been shown in the development of an effective DNA vaccine. However, in addition to the development of an effective DNA vaccine, how protein deficiency affects DNA vaccine-induced immune responses is an important point because protein malnutrition is an indirect cause of immunodeficiency for a significant proportion of children worldwide, for 50% of hospitalized cancer patients, and for people with acquired immune deficiency syndrome. Our study showed that protein deficiency reduced Ag-specific CD8+ T-cell induction, which is crucial for protective immunity. Our study also indicates that nutritional management is important to acquire full protective immunity induced by DNA vaccination.

Acknowledgments

We thank Dr. Nagata for providing the OVA-expression plasmid pCI-OVA.

REFERENCES


28) Mainini ES, McMurray DN. 1998. Protein deficiency


