Areca Nut Extracts Attenuated Interferon-γ and Antigen-specific IgM Production in BALB/c Mice

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ABSTRACT

Areca-nut chewing has been well established as a major risk factor for oral cancer and precancerous diseases, whose pathophysiology has been associated with immune deterioration. Previous studies reported that areca nut extracts (ANE) affected the functionality of lymphocytes and neutrophils *in vitro*. We recently showed that intraperitoneal administration of ANE modulated antigen-specific immunity and promoted inflammatory reactions in ovalbumin (OVA)-sensitized mice. The objective of the present studies was to further investigate the *in vivo* effect of ANE on T cell-mediated immune responses. In non-sensitized mice, a single oral dose of ANE (200 mg/kg) markedly suppressed the production of interferon (IFN)-γ by splenocytes stimulated with the T cell mitogen concanavalin A, whereas the expression of interleukin (IL)-2 or IL-4 was unaltered. In OVA-sensitized mice, daily administration of ANE (200 mg/kg) for 9 days significantly suppressed the antigen-induced production of IFN-γ by splenocytes and the serum level of antigen-specific IgM. Both the cellularity and the metabolic activity of splenocytes were unaffected by the ANE treatment. Collectively, these results demonstrated that oral administration of ANE modulated antigen-specific T cell responses. As IFN-γ is a key cytokine involved in the activation of various immunocompetent cells, ANE-mediated suppression of IFN-γ production may be a critical mechanism contributing to the immune deterioration associated with areca-related oral diseases.

Key words: Areca nut, T-cell, interferon-γ, antigen-specific

INTRODUCTION

Areca quid (AQ) chewing is a popular habit in Taiwan, India, and several other Asian countries with approximately 600 million chewers around the world[1]. AQ chewing has been well established as a major etiological factor for oral cancer and precancerous lesions, including oral leukoplakia and oral submucous fibrosis (OSF)[1,2]. In addition to oral diseases, AQ chewing is also a risk factor of hepatocellular carcinoma[3], suggesting that AQ chewing may cause systemically deteriorating effects with a broad spectrum of outcomes.

To date, the mechanisms responsible for the broad spectrum of areca-associated toxic effects in AQ chewers are mostly unclear. Clinical studies have documented that immune dysfunction is closely associated with the pathophysiology of areca-related oral diseases[4,5]. For example, daily consumption of AQ has been shown correlated with the deregulation of humoral immunity in OSF patients[6]. In addition, cell-mediated immunity has been reported compromised in patients with areca-associated oral diseases[5].

T helper (Th) cells play a pivotal role in the host immunity against tumor formation[7]. In particular, the Th1 cytokine interferon-gamma (IFN-γ) is a key cytokine involved in both innate and acquired immunity, such as the promotion of Th1 cell differentiation, the activation of natural killer cells, cytotoxic T lymphocytes and macrophages, and the stimulation of antibody production[8]. Notably, immunohistochemical studies revealed that Th cells are the major immunocompetent cells present in OSF tissues as evidenced by an elevated ratio of CD4^{+} to CD8^{+} cells[9,10]. However, the expression of IFN-γ in the OSF tissues was suppressed, indicating a down-regulation of T-cell functionality[10]. Concordantly, a decreased ability of peripheral blood mononuclear cells isolated from OSF patients to produce IFN-γ was also observed[11]. Collectively, these lines of evidence demonstrates that the reactivity of T cells, such as the expression of IFN-γ, is down-regulated, which may contribute to

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the dysfunction of cellular immunity in areca-associated oral diseases.

AQ is generally comprised of areca nut (*Areca catechu*), piper betle leaf (*Piper betle*), and lime paste[1]. As clinical reports have shown alterations in many aspects of the immune system in areca-associated oral diseases, the direct influence of areca nut extract (ANE) ingredients on immune cells is recently under intensive investigation. It has been reported that ANE inhibited the phagocytosis and bactericidal activity of neutrophils[12]. The inhibition by areca ingredients on T-cell activation has also been demonstrated[13]. We previously showed that ANE inhibited the metabolic activity and IFN-γ production by murine splenocytes *in vitro*[14]. In addition, ANE enhanced the production of inflammatory cytokines in peripheral blood mononuclear cells[15]. Intraperitoneal administration of ANE promoted inflammatory reactions associated with delayed-type hypersensitivity and modulated antigen-specific antibody production in ovalbumin (OVA)-sensitized mice[16]. Together these studies clearly demonstrate that lymphocytes are one of the sensitive targets to ANE. However, evidence pertaining to the *in vivo* effect of ANE on antigen-specific immunity remains limited and to be further addressed.

The objective of the present study is to investigate the effects of oral administration of ANE on antigen-induced T cell responses. As AQ is consumed by chewing, the oral route of administration is a more relevant mode of exposure compared to the intraperitoneal route. We report here that oral administration of ANE markedly attenuates the expression of the Th1 signature cytokine IFN-γ in both non-sensitized and antigen-sensitized mice. In addition, ANE suppresses the serum production of antigen-specific immunoglobulin M (IgM), substantiating the modulatory effect of ANE on acquired immunity.

MATERIALS AND METHODS

I. *Reagents and Areca Nut Extract*

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Areca nuts were extracted with water as previously described[17], and the extracts were freeze-dried as ANE. The ANE has been confirmed endotoxin-free using a commercial Limulus amebocyte lysate assay kit (Kinetic-QCL®; Lonza Walkersville Inc., Walkersville, MD, USA). Fetal bovine serum (FBS) and RPMI 1640 medium were from Hyclone (Logan, UT, USA). Reagents for ELISA were from BD Biosciences (San Diego, CA, USA).

II. *Animals*

Male BALB/c mice, 5 - 6 weeks of age were purchased from the Animal Breeding Center of the National Taiwan University Hospital (Taipei, Taiwan). On arrival, the mice were randomized, transferred to plastic cages containing a saw-dust bedding (five mice per cage) and quarantined for at least one week. The animal was housed in a temperature (22 ± 2°C), humidity (50 ± 20%), and light-controlled environment (12 h light/dark cycle) with free access to standard laboratory food and water.
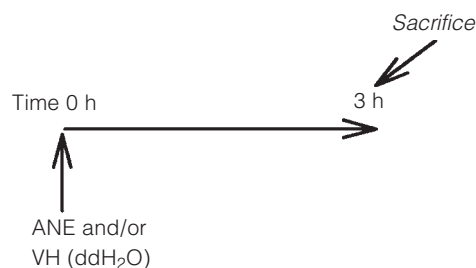
III. *ANE Administration Protocol*

(I) *Non-Sensitized Protocol*

The mice were either left untreated (naïve; NA), or administered by gavage with a single dose of ANE (200 mg/kg; 0.2 mL/mouse) and/or H_2O (vehicle; VH; 0.2 mL/mouse). The mice were sacrificed 3 h post drug administration and their splenocytes were isolated for further experiments (Figure 1A).
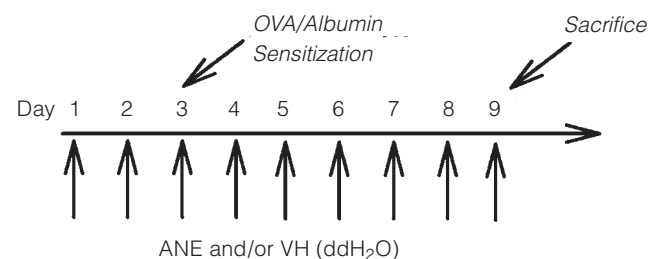
(II) *OVA-Sensitized Protocol*

The mice were either left untreated (NA), or sensitized by intraperitoneal injection with a sensitization solution containing OVA plus alum (100 μg/1 mg; OVA) on day 3. The OVA-sensitized mice were administered once per day

(A) Non-sensitized protocol

(B) Ovalbumin (OVA)-sensitized protocol

Figure 1. Protocols for OVA-sensitization and ANE administration to BALB/c mice. (A) In the non-sensitized protocol, male BALB/c mice were randomly divided into the following groups: naïve (NA), vehicle (VH; ddH$_2$O) and ANE-treated (200 mg/kg). The mice were administered with VH and ANE by gavage. The splenocytes were isolated 3 h after ANE administration for further experimentation. (B) In the OVA-sensitized protocol, mice were randomly divided into the following groups: naïve (NA), OVA-sensitized (OVA), vehicle-treated and OVA-sensitized (VH), and ANE-treated (200 mg/kg) and OVA-sensitized (ANE). Except for the NA group, mice were sensitized with OVA plus alum (100 μg/1 mg) on day 3. ANE and VH were administered daily by gavage from day 1 - 9. All mice were sacrificed 3 h after the final drug treatment, and their serum and splenocytes were obtained for further experimentation.

with ANE (200 mg/kg; 0.2 mL/mouse; ANE) and/or H_2O (VH; 0.2 mL/mouse) by gavage from day 1 - 9. All mice were sacrificed 3 h after the final dose of ANE, and their serum and splenocytes were isolated for further experiments (Figure 1B). The animal experiments were approved by the Institutional Animal Care and Use Committee of the National Taiwan University.

IV. *Spleen Index*

After sacrifice, the spleen of each mouse was dissected out and weighed immediately. The spleen index was calculated as the spleen weight (mg) per body weight (g).

V. *Cellularity of Splenocytes*

Splenocytes were stained with rat anti-mouse CD4 conjugated with fluoroscein isothiocyanate (FITC), rat anti-mouse CD8 and B220 conjugated with PE-Cy5 antibodies (BioLegend, San Diego, CA, USA) in phosphate-buffered saline (PBS) containing 2% FBS. After washing, the single cell fluorescence of 10,000 cells for each sample was measured by a flow cytometer (BD FACSCalibur, San Jose, CA, USA). Data were analyzed using the software Flowjo 5.7.

VI. *Measurement of The Metabolic Activity of Splenocytes*

Splenocytes were cultured in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 5% heat-inactivated FBS. In all cases, splenocytes were cultured at 37°C in 5% CO_2. Splenocytes (5×10^6 cells/mL) were cultured in quadruplicate in 96-well plates (100 μL/well) and stimulated with concanavalin A (ConA; 5 μg/mL) for 48 h or OVA (50 μg/mL) for 72 h. The metabolic activity of splenocytes was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Splenocytes were cultured 4 more h in the presence of MTT (50 μg/mL) and the formed formazan was dissolved with a lysis buffer (10% SDS in *N,N*-demethylformamide) overnight in the dark. The plate was read on a microplate reader at 570 nm using 630 nm as the background reference.

VII. *Cytokine Measurement*

Splenocytes (5×10^6 cells/mL) were cultured in quadruplicate in 48-well plates (300 μL/well) and stimulated with ConA for 48 h or OVA for 72 h. After stimulation, the supernatants from each well were quantified for interleukin (IL)-2, IFN-γ and IL-4 by standard sandwich enzyme-linked immunosorbent assay (ELISA) as previously described[14].

VIII. *Measurement of OVA-Specific IgM*

ELISA plates were coated with 0.05% OVA in coating buffer (0.1 M $NaHCO_3$) and blocked with 1% bovine serum albumin in PBS containing 0.05% Tween-20 (PBST). After

washing with PBST, serum samples were added into wells and incubated for 2 h. After washing, radish peroxidase-conjugated anti-mouse IgM was added and incubated for 1 h. The wells were washed again and a tetramethylbenzidine solution was added for colorimetric detection on a microplate reader.

RESULTS

I. *ANE Attenuated IFN-γ Production by Splenocytes Stimulated with ConA*

T cell-derived cytokines participate in both humoral and cell-mediated immune responses. We therefore examined the effect of ANE on the expression of IL-2, a pivotal cytokine for T-cell clonal expansion, and IL-4 and IFN-γ that are signature cytokines produced by Th1 and Th2 cells, respectively, in non-sensitized mice. The dose (200 mg/kg) of ANE was used according to previous reports showing the *in vivo* effect of ANE to inhibit mast cell degranulation[18], and to induce the sister chromatin exchange of bone marrow chromosomes[19]. Mice were sacrificed 3 h post a single dose of ANE administration (Figure 1A), and their splenocytes were stimulated with the T-cell mitogen concanavalin A (ConA; 5 μg/mL) for 48 h to induced cytokine expression. The production of IFN-γ was significantly attenuated by the ANE treatment (Figure 2A), whereas IL-2 and IL-4 were unaffected (Figure 2B and C). The effect of ANE on cytokine production may be due to alterations in T cell cellularity and/or activity. Thus, the influence of ANE on the spleen index, cellularity and the metabolic activity of splenocytes was examined. The ANE treatment exerted no effect either on the spleen index, cellularity (Table 1) or the metabolic activity of splenocytes activated by ConA (Figure 2D).

### II. *ANE Attenuated Antigen-Specific IFN-γ Production*

As ANE differentially suppressed ConA-induced IFN-γ production by splenocytes of non-sensitized mice,

Table 1. Effect of ANE on spleen index and splenocyte cellularity

	NA[b]	VH	ANE
Spleen Index [a]	3.7 ± 0.1	4.1 ± 0.2	4.0 ± 0.2
Splenocyte Cellularity (%)[c]			
CD4^{+} Cells	25.9 ± 2.8	23.5 ± 1.8	24.7 ± 2.3
CD8^{+} Cells	9.7 ± 0.2	10.1 ± 0.5	10.2 ± 0.7
B220^{+} Cells	48.9 ± 1.0	48.7 ± 2.7	48.2 ± 1.7

[a]Spleen index was calculated as the spleen weight (mg) per body weight (g). Data are expressed as mean ± SE of four samples and are representative of three independent experiments.
[b]NA: untreated; VH: vehicle-treated and OVA-sensitized and challenged and ANE: ANE-treated and OVA-sensitized and challenged.
[c]The percentage of CD4^{+}, CD8^{+} and B220^{+} cells was determined by flow cytometry. Data are expressed as mean ± SE of triplicate samples pooled from three independent experiments.
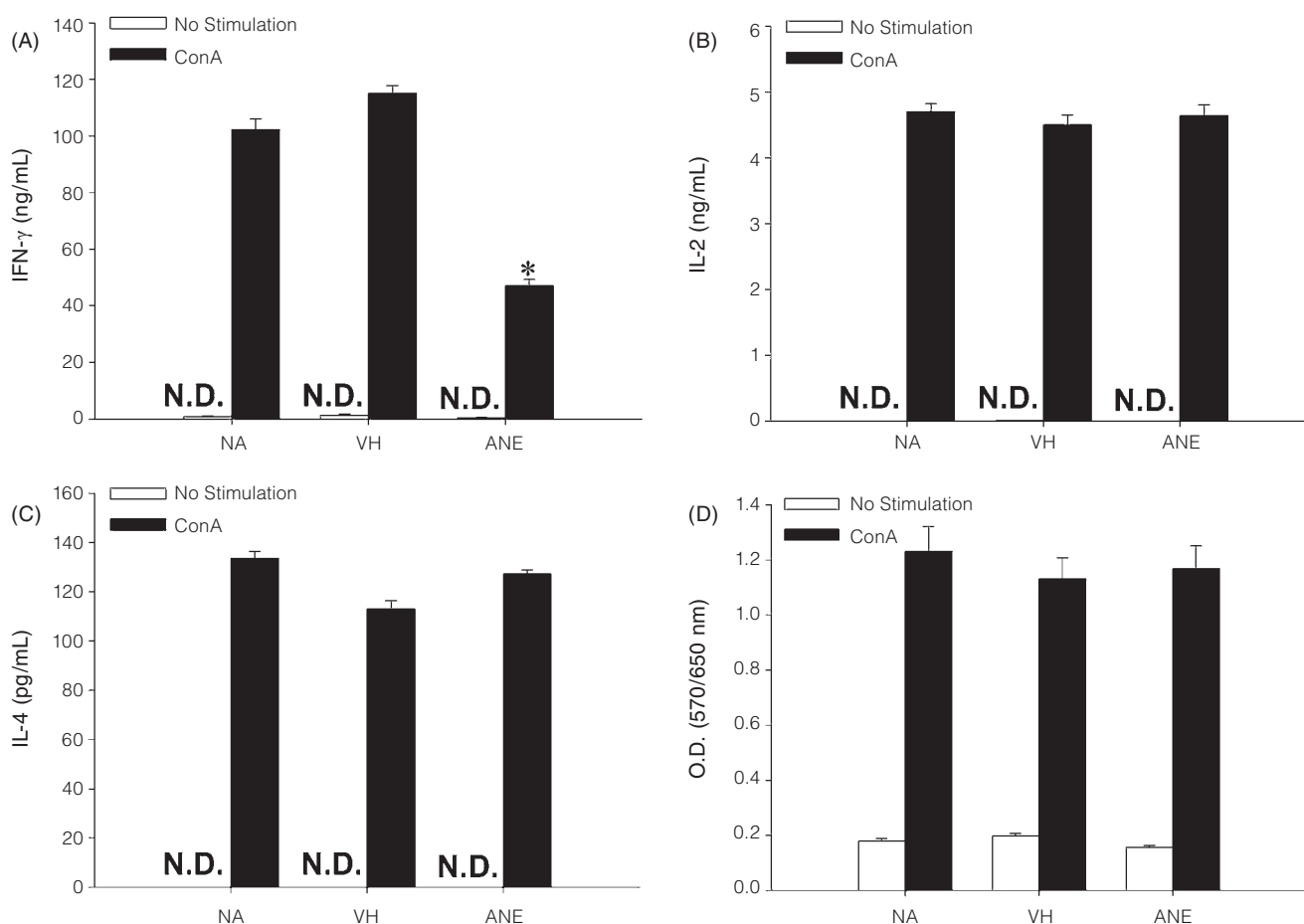
Figure 2. Attenuation of IFN-γ production by ANE administration in non-sensitized mice. Mice were treated with ANE as described in Figure 1A. Splenocytes (5×10^6 cells/mL) were stimulated with concanavalin A (ConA; 5 μg/mL) for 48 h. (A-C) The concentration of IFN-γ, IL-2 and IL-4 in the supernatants was measured by ELISA. (D) The metabolic activity of viable cells was determined using the MTT assay. Data are expressed as the mean ± SE of quadruplicate cultures. Results are representative of six independent experiments. *$p < 0.05$ compared to the VH group.

we thus investigated whether ANE affected antigen-specific IFN-γ production. Splenocytes of OVA-sensitized mice were re-stimulated with OVA (50 μg/mL) in culture for 72 h to induce OVA-specific cytokine production. The OVA re-stimulation successfully induced IFN-γ production (Figure 3; OVA re-stimulation *vs.* no stimulation), which was significantly attenuated by repeated administration of ANE (Figure 3; ANE *vs.* VH). None of the metabolic activity of splenocytes, the spleen index and cellularity was affected by ANE treatment (data not shown).

III. *ANE Attenuated OVA-Specific IgM Production*

Antibody production is one of the hallmark responses in the acquired immunity. Cytokines derived from Th cells play a pivotal role in the activation of antibody-producing B cells. As ANE administration attenuated OVA-specific IFN-γ production, we therefore examined the effect of ANE on the serum level of OVA-specific IgM, the major isotype of immunoglobulin produced during the primary antibody

response. Our data showed a marked increase in the serum OVA-specific IgM in OVA-sensitized mice indicating a successful induction of antibody response, which was significantly attenuated by repeated administration of ANE (Figure 4).

DISCUSSION

Although immune dysfunction has been reported to be associated with the pathophysiology of areca-related oral diseases[5], evidence pertaining to the *in vivo* effect of areca nut constituents on antigen-specific immunity is scarce. The present study demonstrated that oral administration of ANE markedly attenuated the production of IFN-γ by splenocytes in both non-sensitized and antigen-sensitized mice, which is in line with our previous report showing the suppression of IFN-γ production by splenocytes directly exposed to ANE *in vitro*[14]. As one of the major sources for IFN-γ is Th1 cells, these results suggest that ANE differentially affects the

functions of Th1 cells. Furthermore, we showed a marked attenuation of the serum production of antigen-specific IgM in OVA-sensitized mice treated with ANE. On the base of these results, we postulated that the attenuation of the acquired immunity by ANE may contribute to the clinical observed immune dysfunction in patients with areca-related oral diseases[5].

Immune responses mediated by the Th1 signature cytokines IFN-γ are critical for the host defense against microbes and tumor cells[20,21]. IFN-γ can activate the killing activity
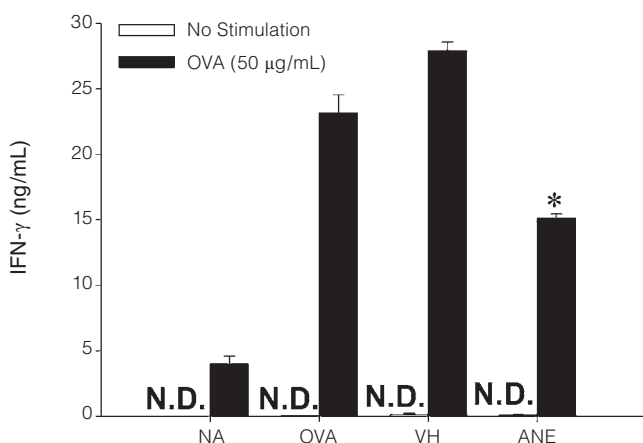
Figure 3. Attenuation of antigen-induced IFN-γ production by ANE administration in OVA-sensitized mice. Mice were treated with ANE as described in Figure 1B. Splenocytes (5×10^6 cells/mL) were stimulated with OVA (50 μg/mL) for 72 h, and the concentration of OVA-specific IFN-γ in the supernatants was measured by ELISA. Data are expressed as the mean ± SE of quadruplicate cultures. Results are representative of four independent experiments. *$p < 0.05$ compared to the matched VH group.
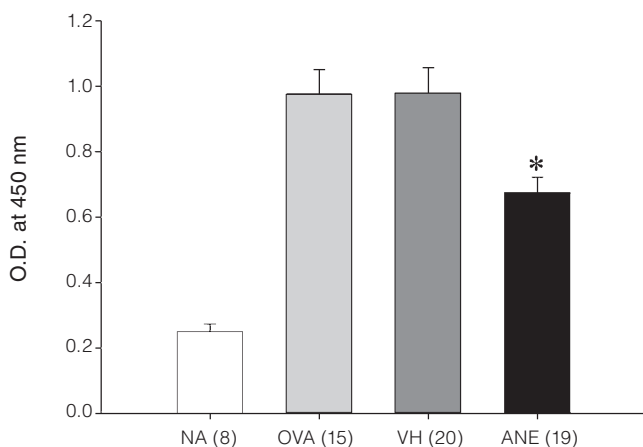
Figure 4. Attenuation of the serum production of OVA-specific IgM by ANE administration. Mice were treated with ANE as described in Figure 1B. The serum titer of OVA-specific IgM was determined by ELISA. Data are expressed as the mean ± SE of pooled data from four independent experiments. The number of individual sample for each group is indicated in the parenthesis. *$p < 0.05$ compared to the matched VH group.

of natural killer cells, cytotoxic T cells and macrophages, and promote the production of opsonizing and complement-fixing antibodies that facilitate the phagocytosis of foreign antigens[20,22]. Lacking IFN-γ in knockout mice increased the incidence of lymphoma, clearly indicating its pivotal role in cancer immunosurveillance[21]. Interestingly, T lymphocytes have been identified as the major immunocompetent cells in the subepithelial connective tissue of OSF patients[9], and the expression of IFN-γ in the oral cavity of OSF patient was suppressed compared to normal controls[23]. Furthermore, the ability of peripheral blood mononuclear cells to produce IFN-γ was diminished in patients with oral leukoplakia, OSF, or head and neck cancers[11,24,25]. Given the critical role of IFN-γ in immunosurveillance, down-regulation of the capability of Th1 cells to express IFN-γ could be a critical mechanism in the pathophysiology of areca-related oral diseases. The disturbances of Th1 cell functions may be caused by immunosuppressive mediators released by tumor cells[26]. Alternatively, as shown in the present studies, contribution from the immunomodulatory effect caused by areca constituents may be another critical mechanism to suppress the functionality of T cells.

An increased production of autoantibodies and circulating immune complexes has been documented in patients with areca-related oral diseases[27,28]. Using the OVA model, we showed that the production of antigen-specific IgM was attenuated by oral administration of ANE, which is consistent with our previous report showing a similar effect by intraperitoneal administration of ANE[16]. It is currently unclear whether ANE attenuated IgM via a direct effect on B cells or an indirect mechanism mediated by cytokine suppression. This issue requires further investigations.

Arecoline at the dose between 5 - 20 mg/kg has been shown to affect splenic cellularity and antigen-specific immune responses[30,31]. However, the amount of arecoline contained in our ANE is < 10 μg/mg of ANE (data not shown). Hence, the dose of arecoline corresponding to 200 mg of ANE/kg is < 2 mg/kg that is much lower than the reported effective dose range of arecoline. Moreover, our data showed that ANE administration did not affect splenic cellularity. Therefore, it is apparent that arecoline may not play a major role in the present studies. In addition to alkaloids, areca nuts contain a significant amount of polyphenols, in particular procyanidins[29]. A growing body of evidence suggests that procyanidins exhibit a broad spectrum of biological effects, such as immunomodulation[32-34]. For example, highly oligomeric procyanidins purified from Jatoba herb ameliorated encephalomyelitis and collagen-induced arthritis in mice via suppression of Th1 immunity, including suppression of IFN-γ production and inhibition of macrophage maturation[33,34]. Therefore, procyanidins might be the active constituents contributing to the immunosuppressive effect of ANE.

In summary, the present studies demonstrated that oral administration of ANE attenuated the production of IFN-γ and OVA-specific IgM. These results indicate that areca nuts contain constituents that can be orally absorbed and produce immunomodulatory effects. Areca-mediated

down-regulation of IFN-γ production may be a potential mechanism involved in the compromised acquired immunity associated with areca-related oral diseases.

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