



Evaluation of the immunosensitizing potential of chlorogenic acid using a popliteal lymph node assay in BALB/c mice

Zhaohua Liu^{a,b}, Zhaoping Liu^b, Yanqiu Shi^b, Gengyin Zhou^{a,*}

^a Department of Pathology, Shandong University School of Medicine, 44 Wen Hua Xi Road, Jinan, Shandong 250012, PR China

^b Center for New Drug Evaluation, Shandong University, 44 Wen Hua Xi Road, Jinan, Shandong 250012, PR China

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ABSTRACT

It has yet to be established whether chlorogenic acid (CGA), a common xenobiotic with potential exposure risk to humans, is associated with immune-mediated hypersensitivity reactions (HRs). The primary limitation in evaluating this potential relationship is the lack of an effective animal model for use in predicting the immunosensitizing potential of low molecular weight compounds (LMWCs). Currently, the popliteal lymph node assay (PLNA) is considered a very promising tool for assessing immunosensitizing potential of LMWCs. To determine whether CGA may possess an intrinsic capacity to stimulate or dysregulate immune responses, and if so, what mechanisms may be involved, we characterized the popliteal lymph node reaction induced by CGA in naive female BALB/c mice using both a direct PLNA (d-PLNA) and a reporter antigen PLNA (RA-PLNA) method. Our results show that CGA failed to induce immunoreactivity following a single subcutaneous injection either alone or when combined with TNP-OVA or TNP-Ficoll. These results indicated that CGA lacks the intrinsic capacity to sensitize or stimulate immune responses in BALB/c mice. Moreover, these results suggest that exposure to CGA may not represent a safety concern for humans and that removal of CGA from Traditional Chinese Medicine Injections may not significantly decrease the prevalence of HRs.

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1. Introduction

Chlorogenic acid (CGA), a natural polyhydric phenol compound, has been identified in medicines, food processing and cosmetic chemicals. CGA-rich plants are found in medicinal plants such as those used as heat-clearing and detoxifying medicinal substances in Traditional Chinese Medicines (TCMs), including *Herba*, *Cortex Eucommiae*, *Flos Lonicerae*, *Herba Houttuyniae* and *Caulis Loniceræ*. CGA is also ubiquitous in edible plants such as apples, sunflowers, coffee, and cacao, which comprise a number of health-promoting foods and beverages consumed world-wide. In addition, CGA is a constituent of a variety of cosmetic products. Accordingly, these wide-ranging applications associated with CGA suggest that it represents a substantial level of exposure to humans.

The significance of such human exposure is related to findings that CGA has a wide array of biological activities. It has been dem-

onstrated that CGA may offer beneficial effects in the treatment of viral infections (Lee et al., 2008), inflammation (Dos Santos et al., 2006), tumors (Jin et al., 2005), and cardiovascular disease (Suzuki et al., 2006). It was also reported that CGA could improve glucose tolerance, decrease plasma lipids (De Sotillo and Hadley, 2002), and reduce the risk of developing type 2 diabetes (Bassoli et al., 2008). In addition, CGA, a known antioxidant, was able to protect erythrocytes (Tang and Liu, 2008) and exert neuroprotective effects (Li et al., 2008b); and chlorogenic laurate, a modified CGA, has a radical-scavenging property which may be used as a preventive antioxidant in liposoluble systems (Xiang and Ning, 2008). Such a variety of effects indicate that CGA is a promising candidate and for use as a potential therapeutic agent.

In spite of the plethora of papers on the pharmacological effects of CGA, systemic safety evaluations of this compound have been seldom studied. It is known that, approximately 6–10% of adverse drugs reactions are attributable to immune-mediated drug hypersensitivity reactions (HRs), and HRs represent the most frequent causes for drug withdrawal from the market due to safety concerns (Ratajczak, 2004). Therefore, there is a great need to investigate the immunosensitizing potential of drug candidates during development. In specific, the immunosensitizing potential of CGA represents an important area of investigation and one that serves as the basis for this report.

Abbreviations: AFCs, antibody-forming cells; CGA, chlorogenic acid; CI, cellularity index; DF, Diclofenac sodium salt; HRs, immune-mediated hypersensitivity reactions; LMWCs, low molecular weight compounds; PB, Phenobarbital; PLNA, popliteal lymph node assay; RA, reporter antigen; STZ, Streptozotocin; TCMs, Traditional Chinese Medicine Injections; TNP-Ficoll, 2, 4, 6-trinitrophenyl-Ficoll; TNP-OVA, 2, 4, 6-trinitrophenyl-ovalbumin; WI, weight index.

* Corresponding author. Tel./fax: +86 531 88383168.

E-mail address: zhougy_sdu@163.com (G. Zhou).

The prevalence of HRs, which follow treatment with many TCMI particularly with Traditional Chinese Medicine Injections (TCMIs), appears to have increased significantly in China over the past 10 years (Ko, 2004). There are 109 TCMI available in Chinese domestic markets. Most TCMI are intravenous injections used in patients suffering from acute and severe disease, which broke from the limitations of the Traditional Chinese Medicine regiments. A small number of TCMI may be injected in via intramuscular or subcutaneous routes. The over 60 year history of applications and enormous use in humans (more than 400 million patients were injected with different TCMI in 2008) have demonstrated that TCMI are playing an important role in the treatment of conditions ranging from cardiovascular and cerebrovascular diseases, respiratory diseases and cancer. Therefore, TCMI are not simple crude extracts, rather they are products that combine Traditional Chinese Medicine theory with modern production technology, and conform to the Good Manufacturing Practice (GMP) standard to be approved for sale by State Food and Drug Administration (SFDA). However, TCMI do contain complex mixtures with a number of unknown constituents and identifying the allergen in these complex mixtures is a key factor in investigating the HRs induced by TCMI. Notably, a high incidence of adverse drug effects usually occurs in TCMI containing high CGA, such as with injections of Yuxingcao and Shuanghuanglian <http://www.sda.gov.cn/WS01/CL0078/38014_1.html>. For this reason most investigators speculated that the cause of these serious adverse reactions were at least in part attributable to CGA. In support of such speculation are previous results showing that polyphenolic antioxidants such as rutin and CGA could enhance IgE production (Gong et al., 2004). Moreover, CGA could combine with human serum albumin (Kang et al., 2004) and this combination has the potential to form a hapten-carrier compound that could elicit a specific immune response.

The limited number of reports performed on immunosensitizing potential of CGA has yielded contradictory results (Anon, 2002; Almeida et al., 2008; Freedman, 1964; Layton et al., 1968). Since CGA is a low molecular weight compound (LMWCs) it remains difficult for regulatory safety studies to predict the potential for LMWCs to induce HRs (Bala et al., 2005). The major problem in identifying immunosensitizing effects of CGA is due to the lack of available animal models to test for this eventuality (Pieters, 2007). At present, the popliteal lymph node assay (PLNA) is regarded as a promising and unique approach to indicate immunosensitization by LMWCs and the potential for use as a screening tool in immunotoxicological hazard identification (Lovik et al., 2007; Pieters, 2007; Ravel and Descotes, 2005; Suda et al., 2000). This new test model is designed on the basis that HRs induced by LMWCs are initiated and maintained by T cells (Zanni et al., 1998). Thus, the detection of lymph node hyperplasia and the reactions mediated by specific antigen receptor B and/or T cells can serve to measure and assess the potency of LMWC to stimulate the immune system. In this way, PLNA can be used to determine whether CGA may have the intrinsic capacity necessary to stimulate or dysregulate immune responses and suggest a mechanism(s) involved in this effect. Five different versions of the PLNA method have been proposed: the direct, secondary, adoptive, reporter antigen (RA) and adjuvant PLNA (Lovik et al., 2007). In this report, we characterized the PLN reactions induced by CGA in naive BALB/c mice using the direct PLNA (d-PLNA) and RA-PLNA. These two methods were selected since d-PLNA could evaluate the existence of a dose–response relationship and RA-PLNA could verify the potential, or not, to sensitize T-lymphocytes.

2. Material and methods

2.1. Animals

Specific pathogen-free female BALB/c mice (6–8 weeks old at the onset of the experiments, mean $\pm$ SD body weight = 20.2 ± 1.84 g) were obtained from the Shandong University laboratory animal center (China) and randomly assigned to their

respective treatment groups. Mice were allowed to acclimate for 1 week and were maintained in polycarbonate cages with wood chips as bedding. Throughout the experiment, mice were housed at a mean fixed temperature of 23 ± 2 °C, relative humidity of $50 \pm 5\%$, and under a 12 h light/dark cycle. Mice had free access to standard diet and drinking water. These experiments were performed in accord with the guidelines of the animal experiment committee of the Veterinary Faculty of the medical school at Shandong University.

2.2. Chemicals and reagents

The test compound, CGA, was purchased from ACROS Organics (CAS: 327-97-9; the purity of CGA was 99% from coffee seeds). The positive control chemicals included Streptozotocin (STZ) from AMRESCO (CAS: 18883-66-4) and Diclofenac sodium salt from Cayman Chemical (USA). The negative control chemical, Phenobarbital, was obtained from International Laboratory USA (CAS: 50-06-6). Dimethyl sulfoxide (DMSO, cell culture grade) was obtained from Solarbio. The reporter antigens TNP-OVA (2,4, 6-trinitrophenyl-ovalbumin), TNP-Ficoll (2,4, 6-trinitrophenyl-Ficoll) and TNP-BSA (TNP-bovine serum albumin) were purchased from BioSearch Technologies (USA).

The antibodies (Abs) used for surface or proliferative marker staining in tri-colour flow cytometry were purchased from the Biotend Company and included: PE/Cy5 anti-mouse CD8a, PE anti-mouse CD4, FITC anti-mouse CD3, FITC anti-mouse CD25, PE anti-mouse CD23, PE/Cy5 anti-mouse CD45R/B220.

Goat anti-mouse IgM (μ chain specific), IgG1 (γ 1 chain specific), IgG2a (γ 2a chain specific) and IgE used in the Elispot assay were purchased from Southernbiotech (USA). The Elispot plate (96-well filtration plate, Multiscreen HTS™ IP sterile plate) was obtained from Millipore (USA); the Elispot reagent kits and AEC coloring system were obtained from the Dakewe Biotech Company Limited (China).

2.3. Direct PLNA and reporter antigen PLNA

The d-PLNA and RA-PLNA were performed as described previously (Carey et al., 2006; Gutting et al., 1999). Briefly, for the direct PLNA, naive mice were injected with a single dose of all the test compounds in the following quantities: 0.75 mg Diclofenac sodium salt, 0.5 or 1 mg CGA, 1 mg Phenobarbital dissolved in 20% DMSO/saline, 1 mg STZ dissolved in saline. For the RA-PLNA, TNP-OVA or TNP-Ficoll, dissolved in PBS and test compounds in vehicle according to the manufacturer's instructions. Mice were injected with each RA (10 μ g) plus a test compound in quantities of: 0.75 mg Diclofenac sodium salt, 1 mg CGA or PB dissolved in 20% DMSO/saline, 1 mg STZ dissolved in saline. All solutions, with the exception of solutions in DMSO, were prepared fresh before use in the experiment and filtered to sterilize.

At the start of the experiment (day 0), right hind footpads of mice were swabbed with 70% ethanol and received a single subcutaneously injection of the test chemical in a 50 μ l volume in the heel-toe direction using an insulin syringe (BD Company) with a 29-gauge needle. On day 7, mice were sacrificed by cervical dislocation. PLNs from both treated and untreated sides were removed and placed in ice-cold PBS/1% BSA. After removal of excess fatty tissue and weighing (0.1 mg analytic balance, Swiss), single cell suspensions were prepared. Cells were washed and centrifuged twice with PBS/1%BSA and then resuspended in 0.5 ml Lympho-Spot™ serum free rodent medium (DKW34-R0100, China). An automatic cell counter was used to determine the cell cellularity of individual PLNs (CYTORECON™, USA). The mean PLN weight index or mean cell count index of each mouse was calculated by dividing the weight or the cell count of the treated (right) side by that of the untreated (left) side. Viability was occasionally checked by trypan blue exclusion and always exceeded 95%.

2.4. Flow cytometry

Simultaneous three-color analysis of cells from each isolated PLN was performed on flow cytometer (FACSCalibur™) using CELLQuest™ software (Becton Dickinson). Cells were centrifuged, resuspended and adjusted to 1×10^5 cells/100 μ l-cells/ml in PBS/1% BSA. The 100 μ l-cell suspensions were incubated with predetermined dilutions of FITC-, PE-, and CY-conjugated mAbs in darkness at 4 °C for 30 min. Finally, after being washed two times using PBS, cells were resuspended in 1% paraformaldehyde, protected from light and stored at 4 °C until analysis (Nierkens et al., 2005b).

2.5. Enzyme-linked immunospot (Elispot) assay

Elispot plates were pre-wetted with 15 μ l PVDF coating buffer (Magi™, Dakewe), pre-coated with 10 μ g/ml TNP-BSA in pyrogen-free phosphate-buffered saline (PBS) (overnight, 4 °C), and then blocked with blocking buffer (37 °C) for 1 h. Single cell suspensions were prepared in serum free medium for rodents (DKW34-R0100, China). The 50 μ l-cell suspensions were placed in one well of a 96-well plate and incubated at 37 °C (5% CO₂ + 95% air) for 4 h. After being aspirated and washed with deionized H₂O three times to lyse cells, diluted HRP (horseradish peroxidase) was added- conjugated with anti-mouse Abs: IgM (1:4000), IgG₁ (1:1000), IgG_{2a} (1:1000), IgE (1:500) to each well and incubated at 4 °C for 18 h.

Thereafter, each well was washed 10 times using washing buffer, with the last wash using PBS. Each well was then added to AEC coloring agents (DKW02-0001, China) in the dark. When the spots were developed, the color reaction was stopped. The plates were dried at room temperature and then analyzed by counting the spots with an automated Elispot reader (Bioreader 4000, German). The results were expressed as the number of AFCs (antibody-forming cells) per recovered PLN.

2.6. Statistics analysis

The values are represented as mean $\pm$ SD. A twofold increase in PLN average weight index (WI ≥ 2) or a fivefold increase in PLN average cellularity index (CI ≥ 5) was established as a positive response. Logarithmic transformations were performed to establish homogeneity of variance for TNP-specific AFC counts. Multiple comparisons of group means were analyzed using a one-way ANOVA with Fisher's least significant difference (LSD) for justified post hoc pair-wise comparisons. A value of $P < 0.05$ was used for results to be considered statistically significant. All statistical analyses were carrying out by SPSS software (version13.0 for windows).

3. Results

3.1. Effect of CGA on PLN weight and cellularity in d-PLNA and RA-PLNA

Weight and cellularity of the PLNs were determined on day 7 after a single subcutaneous injection. Both CGA and Phenobarbital failed to induce a marked increase in average PLN weight (mg) and in PLN average cellularity ($\times 10^6$). However, positive control chemicals such as STZ and Diclofenac sodium salt resulted in a statistically significant enlargement in PLN ($P < 0.05$ and $P < 0.01$, respectively) for both d-PLNA and RA-PLNA treatments. Similarity the average weight index or cellularity index in mice treated with CGA and Phenobarbital did not achieve the criterion established for consideration of a positive response, but in those mice given positive control chemicals, weight index and cellularity index averages were increased two- and fivefold, respectively Tables 1 and 2. In addition to these increases in cell counts, there were large proliferating lymphocytes in PLN cell suspensions treated with positive control chemicals; no such structures were observed in mice treated with CGA or Phenobarbital Fig. 1A–D. Although 1 mg CGA was capable of increasing the number of cells in treated lymph nodes for d-PLNA and RA-PLNA suspensions as compared with that of untreated lymph nodes ($P < 0.05$), the cellularity index failed to achieve the criterion of a positive PLN response (≥ 5 , Table 2).

3.2. Effect of CGA on cell phenotype and biomarker of PLN cell suspensions in d-PLNA

The cell phenotypes, proliferation and activated marker of PLN cell suspensions, were determined by flow cytometry. No statistically significant changes induced by the test compounds were ob-

tained in the percent of T cells ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$) or B cells ($B220^+$), however, STZ increased the percent of $CD3^+CD8^+$ T cells ($P < 0.05$, Fig. 2A). These results suggest that CGA did not affect the subpopulation of lymphocytes in PLN. Moreover, there were no significant differences in percent of $CD25^+$ or $B220^+CD23^+$ positive cells exposed to CGA as compared to those injected with Phenobarbital, which indicated that no T or B cells were activated. In contrast, STZ and Diclofenac sodium salt slightly increased $CD25^+$ and $B220^+CD23^+$ positive cells Fig. 2B.

3.3. Effect of CGA on Anti-TNP-specific AFCs per recovered PLN in RA-PLNA

The production of anti-TNP-specific AFCs IgM, IgG₁, IgG_{2a} and IgE single cell suspensions on the PLN treated side was assessed by the Elispot assay. A spot with circular, densely granulated foci represented an anti-TNP-specific positive AFC. Applications of positive chemicals induced a number of spots; negative chemicals induced few or no spots Fig. 3A and B. Neither CGA nor Phenobarbital significantly increased the number of anti-TNP-specific AFCs to TNP-OVA or TNP-Ficoll. In contrast, STZ predominantly induced the production of anti-TNP IgM and IgG_{2a}, but failed to increase IgG₁ and IgE, whereas Diclofenac sodium salt caused an increase in the number of anti-TNP IgM, IgG₁ and IgE AFCs but not IgG_{2a} Fig. 3C,D.

4. Discussion

The primary issues addressed in the present study were to explore the immunosensitizing properties of CGA and attempt to understand some of the immunological mechanisms responsible for eliciting the specific PLN responses. Our results demonstrate that CGA failed to induce any significant immunoreactivity following a single subcutaneous injection alone or when combined with RA (TNP-OVA or TNP-Ficoll) in naive female BALB/c mice, that is, CGA does not show any intrinsic capacity to sensitize or stimulate immune responses. In support of this conclusion are the data showing that CGA failed to induce an increase in PLN weight and cellularity, relative to animals treated with negative control chemicals, in the d-PLNA on day 7 post-injection. In addition, no significant changes in lymphocyte phenotype were observed in PLN treated mice receiving CGA and the surface proliferation and activation markers, CD23 and CD25, were not increased. Although there was an increase in PLN cellularity when co-injected with TNP-Ficoll or TNP-OVA, production of TNP-specific AFCs treated with CGA in the Elispot assay was similar to that of control levels. Taken together, these findings indicate that CGA could neither

Table 1
Popliteal lymph node weight and cellularity in d-PLNA.

Group	PLN weight (mg)		PLN cellularity ($\times 10^6$)	
	Right/left	WI	Right/left	CI
1 mg PB	2.26 $\pm$ 0.42/1.85 $\pm$ 0.47	1.26	0.68 $\pm$ 0.24/0.43 $\pm$ 0.39	2.24
1 mg STZ	4.28 $\pm$ 1.40 [*] /1.86 $\pm$ 0.67	2.60 ^a	2.15 $\pm$ 0.76 ^{**} /0.46 $\pm$ 0.16	5.32 ^b
0.75 mg DF	4.20 $\pm$ 1.69 [*] /2.09 $\pm$ 0.81	2.49 ^a	2.06 $\pm$ 0.72 ^{**} /0.47 $\pm$ 0.30	5.64 ^b
0.5 mg CGA	2.28 $\pm$ 0.50/1.84 $\pm$ 0.64	1.30	0.92 $\pm$ 0.25/0.59 $\pm$ 0.28	1.98
1 mg CGA	2.73 $\pm$ 0.96/2.10 $\pm$ 0.65	1.43	0.97 $\pm$ 0.51/0.46 $\pm$ 0.23	2.72

Each group of female BALB/c mice was injected a single dose CGA or control chemicals Phenobarbital(PB), STZ, Diclofenac sodium salt(DF) on day 0. On day 7, the PLNs were excised. Single cell suspensions of PLN were prepared. PLN weight (mg) and cellularity ($\times 10^6$) were determined. Results were expressed as mean $\pm$ SD. $n = 6$ animals in each group.

^{*} $P < 0.05$ indicates significant differences compared to the negative control PB.

^{**} $P < 0.01$ indicates significant differences compared to the negative control PB.

^a Weight index (WI) above 2.

^b Cellularity index (CI) above 5 is defined as a positive PLN response.

Table 2
Popliteal lymph node cellularity in RA–PLNA.

Group	PLN cellularity with TNP–OVA ($\times 10^6$)		PLN cellularity with TNP–Ficoll ($\times 10^6$)	
	Right/left	CI	Right/left	CI
1 mg PB	1.03 $\pm$ 0.36/0.45 $\pm$ 0.22	3.08	1.10 $\pm$ 0.49/0.62 $\pm$ 0.30	2.04
1 mg STZ	3.69 $\pm$ 1.61 ^{**} /0.64 $\pm$ 0.12	5.81 ^a	2.91 $\pm$ 1.49 ^{**} /0.54 $\pm$ 0.26	6.77 ^a
0.75 mg DF	3.03 $\pm$ 1.76 [*] /0.62 $\pm$ 0.22	5.49 ^a	2.89 $\pm$ 1.23 [*] /0.65 $\pm$ 0.23	5.11 ^a
1 mg CGA	1.90 $\pm$ 1.10/0.48 $\pm$ 0.19	4.16	1.71 $\pm$ 0.86/0.54 $\pm$ 0.28	4.26

Each group of female BALB/c mice was co-injected with 10 μ g TNP–OVA or TNP–Ficoll on day 0 combined with: (1) Phenobarbital (PB); (2) STZ; (3) Diclofenac sodium salt (DF); or (4) CGA. On day 7, the treated side PLNs were excised. Single cell suspensions of PLN were prepared. PLN cellularity ($\times 10^6$) was determined. Results from 2 separate experiments are presented and expressed as mean $\pm$ SD. $n = 6$ animals in each group.

^{*} $P < 0.05$ indicates significant differences compared to the negative control PB.

^{**} $P < 0.01$ indicates significant differences compared to the negative control PB.

^a Cellularity index (CI) above 5 is defined as a positive PLN response.

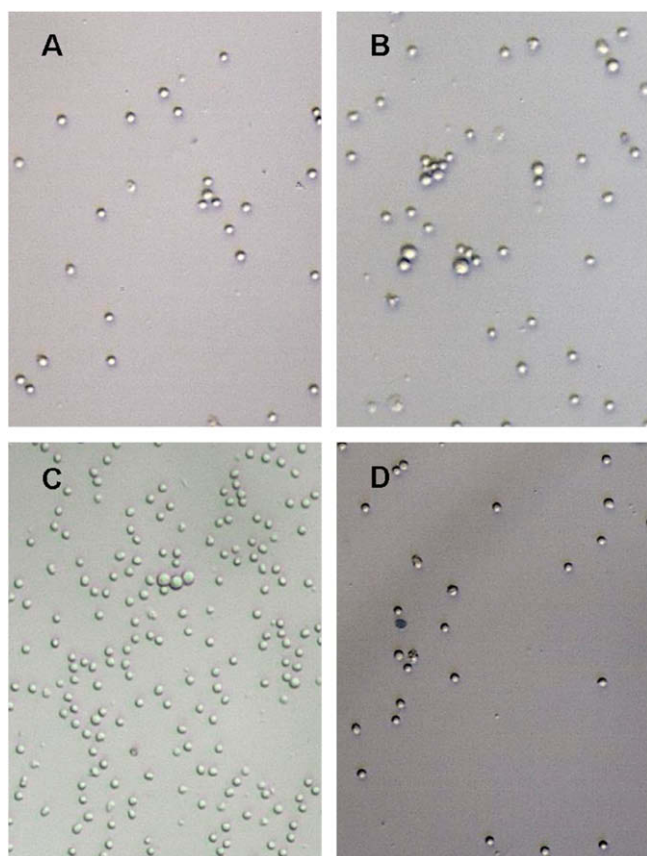


Fig. 1. Photographs of cell suspensions from the treated side PLN in the PLNA. Each group of female BALB/c mice was injected with a single CGA dose or control chemical (Phenobarbital, STZ or Diclofenac sodium salt) on day 0. On day 7, the PLNs were excised and PLN cellularity was determined. STZ and Diclofenac sodium salt exposure caused an elevation in numbers of PLN cells and proliferating large lymphocytes. Photographs were taken from a CytoreconTM automatic cell counting machine with a one-fold magnification ratio. Cell viability treated with Phenobarbital or CGA was verified by trypan blue exclusion and both exceeded 95%. A, 1 mg Phenobarbital (PB); B, 1 mg STZ; C, 0.75 mg Diclofenac sodium salt (DF); D, 1 mg CGA.

stimulate an immune response in a nonspecific manner nor specifically sensitize T cells, thus suggesting that CGA does not possess any immunosensitizing potential in BALB/c mice.

Prior preclinical tests used to assess CGA capacity to affect immune responses have employed guinea pig models which have a number of inherent problems. For example, the use of Freund's adjuvant, the need for recipient animals to display passive cutaneous anaphylaxis reactions, the use of a hapten-carrier conjugate for

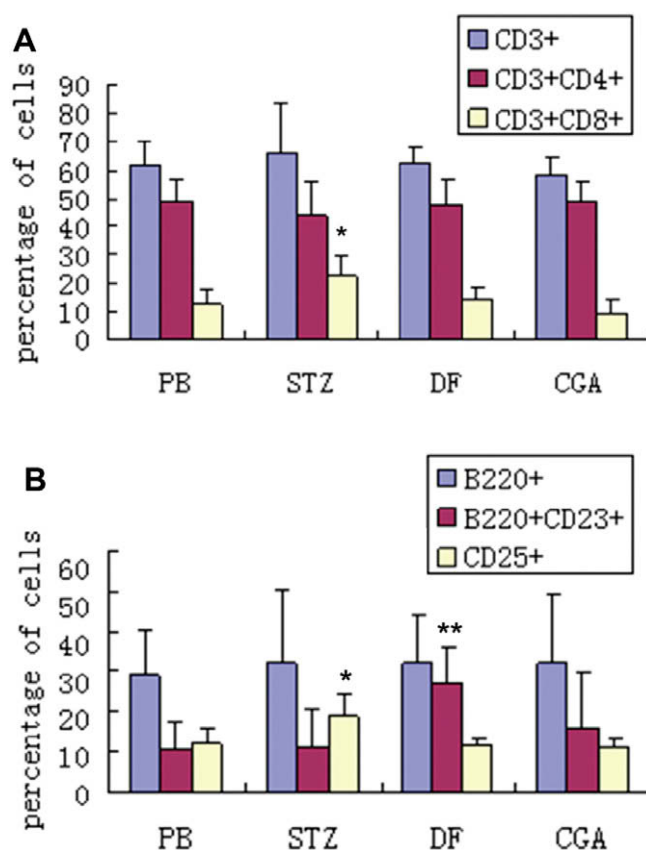


Fig. 2. Tri-color flow cytometric analysis of treated side PLN cell suspensions in d-PLNA. Each group of female BALB/c mice was injected with a single dose: (1) 1 mg Phenobarbital (PB); (2) 1 mg STZ; (3) 0.75 mg Diclofenac sodium salt (DF); or (4) 1 mg CGA on day 0. On day 7, the treated side PLNs were excised. Single cell suspensions of PLN were prepared. Cell percentage of total T (CD3⁺), T helper (CD3⁺CD4⁺), T cytotoxic (CD3⁺CD8⁺), total B (B220⁺) cells and expression of proliferation marker (CD25⁺) and B activated marker (B220⁺CD23⁺) were analyzed. Results are expressed as a percentage change from the mean $\pm$ SD. $n = 6$ animals in each group. ^{*} $P < 0.05$ and ^{**} $P < 0.01$ indicate statistically significant differences compared to the negative control Phenobarbital. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

an eliciting antigen, a long immunization schedule, a complicated protocol, and poor predictability of LMWCs allergenicity all contribute to the ineffectiveness of this method (Aida et al., 1998; Weaver et al., 2003). The murine local lymph node assay (LLNA) is a validated technique developed for evaluating the sensitization potential of chemicals following dermal exposure, but is not effective with regard to detecting the risk of sensitization after systemic

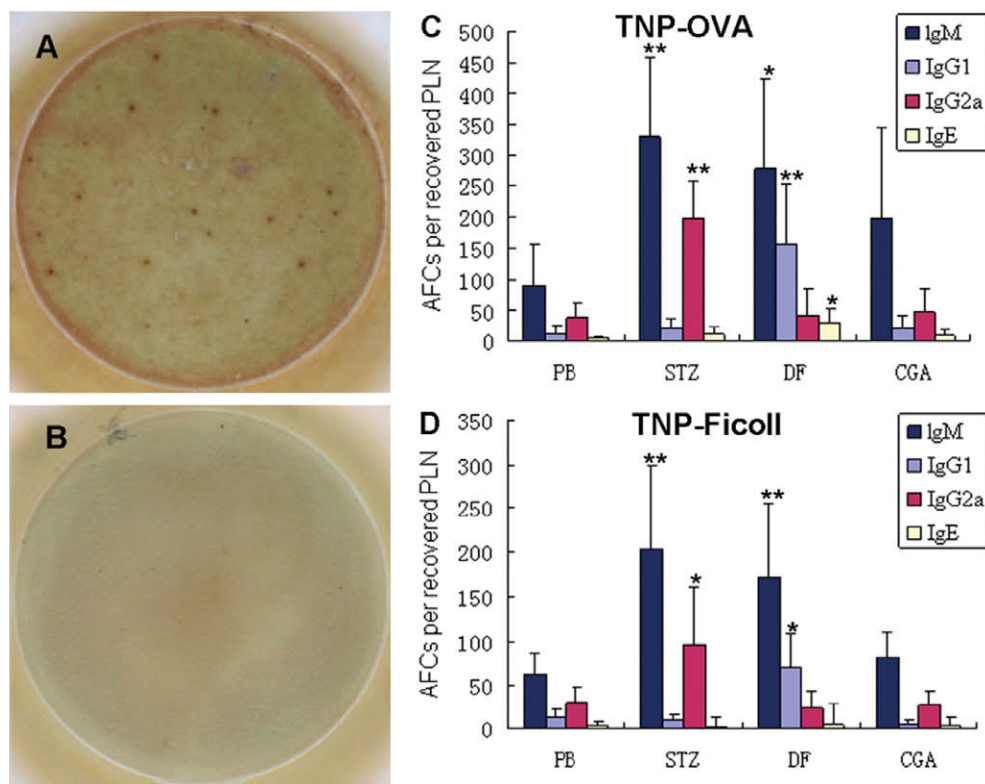


Fig. 3. Detection of anti-TNP-specific AFCs by Elispot assay. Each group of female BALB/c mice was co-injected with 10 μ g TNP-OVA or TNP-Ficoll on day 0 combined with: (1) 1 mg Phenobarbital (PB); (2) 1 mg STZ; (3) 0.75 mg Diclofenac sodium salt (DF); or (4) 1 mg CGA. On day 7, the treated side PLNs were excised. Single cell suspensions of PLN were prepared. IgM, IgG1, IgG2a and IgE were assessed using Elispot assay. Positive chemicals produced AFCs spots with circular, densely granulated foci; CGA and Phenobarbital produced few or no spots (A, B). Results from 2 separate experiments are presented and expressed as AFCs/recovered PLN (mean $\pm$ SD). $n = 6$ animals in each group. C, test chemicals mixed with 10 μ g TNP-OVA; D, test chemicals mixed with 10 μ g TNP-Ficoll. $P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences compared to the negative control Phenobarbital.

exposure. Skin sensitization is most commonly a T cell mediated hypersensitivity reaction (HR). There exist several well-established preclinical models, such as the Guinea Pig Maximization Test (GPMT) and Buehler Test (BT) for assessing this type of HR. The LLNA is only an alternative approach to GPMT or BT (Gerberick et al., 2007; McGarry, 2007). Since common signs and symptoms of the adverse drug effects related to TCMI containing CGA, present with the following features: urticaria, rash, angioedema, hypotension or life-threatening shock, investigators speculate that these adverse effects are mainly mediated by IgE. Traditionally, three methods have been used extensively to detect inductions of IgE-mediated HR: (1) the passive cutaneous anaphylaxis (PCA) assay, (2) the active cutaneous anaphylaxis (ACA) assay, and (3) the active systemic anaphylaxis (ASA) assay. However, these assays have not proven useful in identifying small molecular weight allergens <<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM079239.pdf>>. In fact, there are data in the open literature using these methods in guinea pigs, mice or rats as a means to determine the immunosensitizing potential of CGA which all have indicated negative results (Li et al., 2008a; Luo et al., 2009). As negative results in these assays do not necessarily indicate that CGA (one of LMWCs) lacks sensitizing potential, there is a need for establishing new methods to evaluate such effects. There are little data to support the use of the LLNA to detect the non-dermal sensitizing potential of chemicals. In addition, the LLNA requires a radiochemistry laboratory. In the absence of an appropriately equipped laboratory performing a LLNA, as a substitute for guinea pig tests, is economically prohibitive. Therefore, in consideration of the route of exposure (Intramuscular or Intravenous injections), the clinical characteristic (IgE-mediated)

and limitations of our laboratory facilities, we considered that the LLNA may not provide the best approach to evaluate the immunosensitizing potential of CGA within our experiments. By contrast, the PLNA assay has proved to be a reliable, fast and reproducible method to predict the allergenic potential of LMWC drugs (Lovik et al., 2007; Pieters, 2001, 2007; Ravel and Descotes, 2005; Suda et al., 2000). So far, over 130 drugs and chemicals have been tested in one or more variations of the PLNA and their potential to induce lymph node hyperplasia correlated well with documented allergic or autoimmunogenic potential (Pieters, 2001). However, to the best of our knowledge, no studies have been conducted to assess the immunosensitizing potential of CGA with the PLNA. Therefore, the application of this assay not only provides readout parameters that can be measured accurately, but might also be able to overcome the limitations of other animal models used to assess CGA effects.

There exist a number of advantages of the PLNA. The d-PLNA, a widely used and partially validated method, can be easily adapted and applied within standard laboratory protocols (Ravel et al., 2004). Furthermore, it has been shown that flow cytometric analysis of cell phenotypes and proliferation marker expressions can improve the sensitivity of d-PLNA (Tuschl et al., 2002). Therefore, as one approach to validate this method in our laboratory we first screened the immunosensitizing potential of CGA by using d-PLNA combined with the flow cytometric method in response to different doses of CGA. One limitation of the d-PLNA is that it cannot provide any insight into the mechanism of LMWC-induced hypersensitivity. Therefore, a stepwise strategy to determine immune response specificity was developed as achieved with the RA-PLNA method. This approach is particularly useful for a hazard

assessment of the systemic sensitizing potential of pharmaceuticals (Pieters et al., 2007). With the RA–PLNA, test drugs are injected subcutaneously as combined with either the T cell independent antigen, TNP–Ficoll, or the T cell dependent antigen, TNP–OVA. The specific antibody responses are then measured by Elispot assay. A TNP-specific IgG response to TNP–Ficoll demonstrates the activation of neoantigen-specific T cells. Whereas a positive IgG response to TNP–OVA, but not to TNP–Ficoll, reveals that the test drug induced only an inflammatory reaction. In this way, with use of the RA–PLNA, it is possible to differentiate between immunosensitizing (sensitizers), immunostimulating (irritants), and innocent LMWCs (Carey et al., 2006; Gutting et al., 1999; Ravel and Descotes, 2005). Thus, this *in vivo* model may enable one to differentiate between type 2 and type 1 responses which are associated with different types of HRs (Nierkens et al., 2005a; Ravel and Descotes, 2005). In our study, Phenobarbital was used as a negative control since Phenobarbital failed to induce any PLN response while STZ (known to induce type 1 immune responses and provoke diabetes) and Diclofenac sodium salt (known to induce type 2 immune responses and produce anaphylaxis in humans) were used as positive controls. As expected, the immunostimulating agents, Diclofenac sodium salt and STZ significantly increased the weight and cellularity in the d-PLNA as well as the cellularity and production of AFCs in the RA–PLNA in this model. However, there was no difference in PLN reaction when injected with the negative LMWC Phenobarbital. These findings were in concordance to previous reports (Albers et al., 1997; Gutting et al., 1999; Nierkens et al., 2005a) which support our conclusions regarding the interpretation of data collected from the d-PLNA or RA–PLNA. It seems likely that our results as obtained with the d-PLNA and RA–PLNA represented the true nature of LMWCs to CGA *in vivo*. Thus, we cannot only conclude that CGA is not an allergen associated with IgE-mediated HRs in humans (Anon, 2002), but also demonstrate further that it failed to induce non-IgE-mediated HRs.

Although many investigators in China postulated that CGA may lead to serious adverse reactions of TCMI or elicit a specific immune response (Gong et al., 2004; Kang et al., 2004), in fact, there are few experimental data *in vivo* to support this hypothesis. On the contrary, polyphenol compounds in plants such as caffeic acid, CGA and rosmarinic acid have been reported to have antioxidative activities, and thus, were beneficial in the treatment of allergic diseases (Ito et al., 1998). Moreover, data from previous studies have indicated that CGA could reduce ROS levels in antigen–IgE-activated mast cells *in vitro* and inhibit histamine release by these activated mast cells (Chen et al., 2000). Therefore, CGA might also suppress allergic reactions. Analyses of data from the literature confirmed that HRs accounted for about 90% of the adverse reactions related to various types of respiratory TCMI containing CGA <<http://www.cde.org.cn/dzkw.do?method=largePage&id=307>>. Moreover characteristics of patients with HRs induced by TCMI were similarly found in workers in the coffee industry (Freedman, 1964; Bariana et al., 1965). There is little definitive evidence on which to determine the mechanistic bases of CGA-related-HRs, and therefore our first attempts involve speculations based on clinical characteristics. Mechanistic hints from clinical characteristics have indicated that CGA-related-HRs fit into either IgE-mediated or anaphylactoid reactions. Since allergic reactions cannot be distinguished from anaphylactoid reactions it is possible that most adverse reactions induced by TCMI that occur when patients are first exposed to these agents, may not be due to the immune-mediated hypersensitivity resulting from a specific antigen. The term “anaphylactoid reactions” is used to describe a non-immune-related HR to drugs. This type of HR is the result of non-immune mechanisms, including non-immunological activation of the complement system, direct histamine release from mast cells or basophils, interaction with enzymes, and dose and rate of infusion. A variety of drugs such as van-

comycin, opioids or aspirin can induce anaphylactoid reactions. Recent estimates suggest that anaphylactoid reactions which mimic “true allergy” clinically, but are actually not initiated or mediated by pre-existing IgE antibodies, may represent as many as 77% of all HRs. Idiosyncratic drug reaction is also a significant health problem due to its unpredictable nature, poorly understood pathogenesis, and potential to result in fatal outcomes. Most idiosyncratic reactions are immune-mediated and others are related to a pharmacogenetic predisposition which can be characteristic of a few patients. For example, penicillin or carboplatin could induce an IgE-mediated idiosyncratic drug reaction. Therefore only a small percent of drug-related HRs are truly “allergic”, commonly with the production of a specific antibody or sensitized T cells (Szebeni, 2005; Uetrecht, 2007; Uetrecht, 2008). Anaphylactoid reactions are clinically indistinguishable from IgE-mediated reactions as the same mediators are involved. In some patients, TCMI-related HR does arise upon first exposure or is related to a fast mode of infusion which is congruent with the characteristics of anaphylactoid reactions. In fact, it has also been demonstrated convincingly by Han et al., 2009 that HRs induced by Yuxingcao injection are associated with its solubilizer Polysorbate 80 (Zhang et al., 2008). However, some patients may be exposed to widely-existed CGA accidentally via their diet, lifestyle or from environmental sources and therefore are sensitized to CGA. In this condition, the CGA-induced HR may be a specific immune (idiosyncratic) rather than a non-immune related reaction. Therefore, we used the PLNA in this study in order to detect whether CGA-induced HR resulted from an idiosyncratic reaction. These data, as derived from the PLNA, provide support for previous studies (Layton et al. 1968), where it was concluded that CGA was not a major allergen or objectionable constituent in TCMI.

While we have demonstrated the assets of the PLNA method, there remains much work to be performed with this assay. In this study, a maximum dose of 1 mg CGA in 20% DMSO/80% saline per mouse was arbitrarily selected, as this dose is selected by most researchers using the PLNA. However, previous studies demonstrated that there was an optimal concentration for test chemicals in the PLNA (Vial et al., 1997). Perhaps the effectiveness of this method could be enhanced by using different doses in the RA–PLNA (Tuschl et al., 2002). In addition, the subcutaneous injection into the footpad does not represent a normal route of exposure for humans and metabolism within the footpad may be different from that of a systemic exposure in mice or humans. Nor is it known whether CGA metabolites can become an allergen or happen which may then induce HRs. In fact, it has been shown that false-negative PLN responses, induced by some parent molecules of well-known allergic compounds were the result of a deficiency of metabolism within the footpad (Bloksma et al., 1995). It might then be worthwhile to identify the main CGA metabolites *in vivo*, and then test these in the PLNA. These represent a sample of future issues that need to be addressed to maximize the PLNA effectiveness.

In summary, we have shown that CGA does not possess any intrinsic capacity to stimulate or dysregulate immune responses in naive BALB/c mice using the d-PLNA and RA–PLNA. These findings combined with the results of ASA and PCA (Li et al., 2008a; Luo et al., 2009) indicate that CGA failed to produce HRs after systemic exposure in guinea pigs, mice or rats. As a result we propose that removal of CGA from TCMI by itself may not significantly decrease the prevalence of HRs caused by TCMI.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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