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

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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.

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 Lonicerae. 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 demonstrated 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; TCMIs, Traditional Chinese Medicine Injections; TNP–Ficoll, 2, 4, 6-trinitrophenyl–Ficoll; TNP–OVA, 2, 4, 6-trinitrophenyl–ovalbumin; WI, weight index.

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The prevalence of HRs, which follow treatment with many TCMs particularly with Traditional Chinese Medicine Injections (TCMIs), appears to have increased significantly in China over the past 10 years (Ko, 2004). There are 109 TCMIs available in Chinese domestic markets. Most TCMIs 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 TCMIs 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 TCMIs in 2008) have demonstrated that TCMIs are playing an important role in the treatment of conditions ranging from cardiovascular and cerebrovascular diseases, respiratory diseases and cancer. Therefore, TCMIs 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, TCMIs 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 TCMIs. Notably, a high incidence of adverse drug effects usually occurs in TCMIs 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 ± 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 ± 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-color flow cytometry were purchased from the Biolegend 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 (γ1 chain specific), IgG2a (γ2a 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 μg) 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 μl 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 LymphoSpot™ 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 Dickenson). Cells were centrifuged, resuspended and adjusted to 1×10^5cells/100 μl-cells/ml in PBS/1% BSA. The 100 μl-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 μl PVDF coating buffer (Magi™, Dakewe), pre-coated with 10 μg/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 μl-cell suspensions were placed in one well of a 96-well plate and incubated at 37 °C (5% CO_2 + 95% air) for 4 h. After being aspirated and washed with deionized H_2O three times to lyse cells, diluted HRP (horseradish peroxidase) was added- conjugated with anti-mouse Abs: IgM (1:4000), IgG_1 (1:1000), IgG_{2a} (1:1000), IgE (1:500) to each well and incubated at 4 °C for 18 h.