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Glutamine reduces the expression of leukocyte integrins leukocyte function–associated antigen-1 and macrophage antigen-1 in mice exposed to arsenic

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Abstract

Chronic arsenic exposure results in an increased oxidative stress and inflammation in the body. Glutamine (GLN) is an amino acid considered to have immunomodulatory effects and attenuate the inflammatory reaction. This study was designed to examine the effect of GLN supplementation on inflammatory-related leukocyte integrin expression and in vitro splenocyte cytokine production in mice exposed to arsenic. Mice were assigned to the control and experimental groups. The control group drank deionized water, whereas the experimental group drank deionized water containing 50 ppm of sodium arsenite. Each control and experimental group was further divided into 2 subgroups and fed diets for 5 weeks. One subgroup was fed a semipurified diet, whereas the other subgroup was fed a diet where part of the casein was replaced with GLN, which provided 25% of the total amino acid nitrogen. The results showed that plasma GLN levels of mice in the arsenic group were significantly lower than those in the control groups. Glutamine supplementation reversed the depletion of plasma GLN in the arsenic group. β_2 intergins, including leukocyte function–associated antigen-1 and macrophage antigen-1 expressed by leukocytes, were significantly higher in the arsenic group than the control groups. Glutamine supplementation reduced leukocyte integrin expression in mice exposed to arsenic. There were no differences in interleukin 4, interleukin 6, interferon γ, and tumor necrosis factor α production between the 2 arsenic groups when splenocytes were stimulated with mitogen. These results suggest that arsenic exposure results in depletion of plasma GLN and higher leukocyte integrin expression. Glutamine supplementation normalized the plasma GLN levels and reduced leukocyte leukocyte function–associated antigen-1 and macrophage antigen-1 expression. However, cytokine modulation may not be responsible for reducing leukocyte integrin expression in mice exposed to arsenic.

Keywords: Arsenic; Glutamine; Leukocyte function–associated antigen-1; Macrophage antigen-1; Interleukin 4; Interleukin 6; Interferon-γ; tumor necrosis factor α; Mice

Abbreviations: ANOVA, analysis of variance; GLN, glutamine; IFN-γ, interferon γ; IL-4, interleukin 4; IL-6, interleukin 6; LFA-1, leukocyte function–associated antigen-1; LPS, lipopolysaccharide; Mac-1, macrophage antigen-1; PHA, phytohemagglutinin; Th1, T helper type 1; Th2, T helper type 2; TNF-α, tumor necrosis factor α.

1. Introduction

Arsenic is a ubiquitous element widely distributed in the environment. The main source of arsenic exposure for the general population is through ingestion of high-arsenic drinking water. Chronic arsenic exposure is associated with an increased risk of vascular diseases, including ischemic heart disease, cerebrovascular disease, and carotid atherosclerosis [1,2]. The pathogenic mechanism of arsenic atherogenicity is not completely understood. Previous reports have shown that arsenic results in the generation of

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reactive oxygen species both in vivo and in vitro [2-4]. Oxidative stress may have an impact on the atherogenic process by modulating intracellular signaling pathways in vascular tissues affecting inflammatory cell adhesion, migration, and proliferation [5].

Blood leukocytes are mediators of host defenses and inflammation localized in the earliest lesions of atherosclerosis. The initial sign of inflammation is the capture of leukocytes from the bloodstream and their subsequent rolling along the endothelium of postcapillary venules [6]. Patches of arterial endothelial cells express selective adhesion molecules on their surface that bind to various classes of leukocytes [7]. Leukocyte function–associated antigen-1 (LFA-1) and macrophage antigen-1 (Mac-1) are members of the leukocyte adhesion molecules—β_2 intergins (CD18). Leukocyte function–associated antigen-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) are thought to play central roles in mediating the firm adhesion of leukocyte to endothelial cells [8]. Overexpression of adhesion molecules facilitate leukocyte-endothelial interaction, which results in endothelial dysfunction and vascular damage [9]. Synthesis of the adhesion molecules is regulated by various immune modulatory mediators; among them, proinflammatory cytokines play important roles in the expression and activation of the adhesion molecule [10,11].

Glutamine (GLN) is an abundant free amino acid in the plasma and the tissue pool. Previous reports have revealed that GLN has immunomodulating properties [12,13]. A relatively GLN-deficient state is created by the catabolic process, and that GLN supplementation can correct this nutritional deficiency and, hence, improve outcomes [14,15]. Glutamine is required during catabolic processes to manifest optimal tissue responses to catabolism, inflammation, and infection. Glutamine is considered an essential amino acid during certain inflammatory conditions [16,17]. A previous study showed that GLN supplementation attenuated proinflammatory cytokine release and protected against organ damage in a rat model [18]. Also, Arndt et al [19] demonstrated that GLN administration reduced leukocyte adhesion and transmigration in indomethacin-induced intestinal inflammation in the rat. Because chronic arsenic exposure results in an inflammatory reaction in the body, we hypothesize that GLN administration may have effect on decreasing inflammatory-related cytokine production and leukocyte adhesion molecule expressions in mice exposed to arsenic. Therefore, we designed this study to examine the potential impact of GLN intervention on leukocyte integrin expression and in vitro splenocyte inflammatory-related cytokine production in mice with arsenic exposure.

2. Methods and materials

2.1. Animals

Male BALB/c mice aged 4 weeks and weighing 10 to 15 g were used in this study. All mice were housed in temperature- and humidity-controlled rooms and were allowed free access to standard chow for 1 week before the experiment. The study protocol was approved by the Animal Care Committee of Taipei Medical University, Taiwan, ROC (approval no. LAC-91-0094). The care of the animals was in full compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985) as reviewed by the animal care and use committee.

2.2. Study protocol

Forty mice were assigned to the control or experimental groups, 20 mice to each group. Mice in the control group drank deionized water, whereas those in the experimental group drank deionized water containing 50 ppm of sodium arsenite ($NaAsO_2$), which is equivalent to 6 to 8 mg/kg per day. Each control and experimental group was divided into 2 subgroups, with 10 mice in each subgroup. One subgroup was fed a common semipurified diet, whereas the other subgroup was fed the diet where GLN replaced part of the casein, which provided 25% of total amino acid nitrogen (Table 1). The experimental diets were fed for 5 weeks. There were 4 groups in this study: CC group, no arsenic or GLN administered; CG group, no arsenic but supplemented with GLN; AC group, with arsenic but no GLN supplementation; and AG group, with both arsenic and GLN supplementation. Food and water intake was recorded daily during the experimental period. At the end of the study, all mice were anesthetized with an intraperitoneal pentobarbital sodium (50 mg/kg body weight [BW]). After injection of pentobarbital for 15 to 20 minutes, mice became unconscious and were killed by heart puncture.

Table 1
Ingredient and nutrient composition of the semipurified diet (g/kg)

Ingredient	GLN-supplemented group	Control group
Casein	165	220
GLN	45	–
Crude protein	34.4	34.4
Corn starch	667	657
Soybean oil	44	44
Vitamin mixture [a]	10	10
Salt mixture	35	35
Methyl-cellulose	30	30
Choline chloride	1	1
DL-Methionine	3	3

The salt mixture contained the following (mg/g): calcium phosphate dibasic 500, sodium chloride 74, potassium sulfate 52, potassium citrate monohydrate 220, magnesium oxide 24, manganese carbonate 3.5, ferric citrate 6, zinc carbonate 1.6, cupric carbonate 0.3, potassium iodate 0.01, sodium selenite 0.01, and chromium potassium sulfate 0.55.

[a] The vitamin mixture contained the following (mg/g): thiamin hydrochloride 0.6, riboflavin 0.6, pyridoxine hydrochloride 0.7, nicotinic acid 3, calcium pantothenate 1.6, D-biotin 0.02, cyanocobalamin 0.001, retinyl palmitate 1.6, DL-α-tocopherol acetate 20, cholecalciferol 0.25, and menaquinone 0.005.

2.3. Analysis and measurements

2.3.1. Plasma GLN level analysis

Blood samples were collected in tubes containing heparin and immediately centrifuged. Plasma amino acids were analyzed by a standard ninhydrin technology (Beckman Instrument, model 6300, Palo Alto, Calif) after deproteinization of the plasma with 5% salicylic acid [20].

2.3.2. Flow cytometric analysis

To determine the integrin expression on leukocytes, we used 100 μL of whole blood containing fluorescein-conjugated rat anti-mouse CD11a, CD11b (Serotec, Oxford, UK), and phycoerythrin-conjugated rat anti-mouse CD18 (Serotec) to identify LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), respectively. After staining for 15 minutes, 1 mL of red blood cell lyzing buffer (Serotec) was added to lyse the red blood cells and to fix the stained leukocytes. Fluorescence data were collected on 1×10^5 viable leukocytes and analyzed by flow cytometry (Coulter, Miami, Fla). Leukocytes were gated on the basis of forward scatter and side scatter profile and analyzed for the expression of LFA-1 and Mac-1, respectively. The results are presented as a percentage of CD11a- or CD11b-presenting cells in 1×10^5 leukocytes.

2.3.3. In vitro cultures of splenocytes

Splenocytes were obtained by mechanical disruption of the spleen with a spatula on a stainless steel mesh. Cell suspensions were passed through a sterile nylon mesh to remove debris. Red blood cells were lysed using sterile distilled water for 15 seconds and immediately neutralized to isotonic cell suspensions. After washing with phosphate-buffered saline 3 times (300*g* for 5 minutes), splenocytes were resuspended in RPMI-1640 with antibiotics and fetal calf serum. The number of isolated splenocytes was determined by a hemacytometer count using the trypan blue dye exclusion method.

2.3.4. Cytokine assay

Phytohemagglutinin (PHA) (200 ng/mL; Sigma, St Louis, Mo) and lipopolysaccharide (LPS) (1 μg/mL, Sigma) were used to stimulate cytokine production by isolated splenocyte lymphocyte cell cultures. Triplicate wells of 96-well flat-bottomed microtiter plates (Falcon, Becton Dickinson, Oxford, Calif) were seeded with splenocytes (2.5×10^6 cells/mL in RPMI-1640) and mitogen. The control well contained cells plus an equal volume of medium. After incubating PHA or LPS for 24 hours at 37°C in a CO_2 incubator, supernatants were centrifuged and stored at −70°C until analyzed for cytokine concentration. Concentrations of interleukin (IL)-2, IL-4, and interferon (IFN)-γ in PHA-stimulated splenocyte supernatants and IL-6 and tumor necrosis factor (TNF)-α in LPS-stimulated splenocyte supernatants were determined by commercially available enzyme-linked immunosorbent assay kits (Amersham Pharmacia Biotech, Buckinghamshire, UK). Procedures followed the manufacturer's instruction.

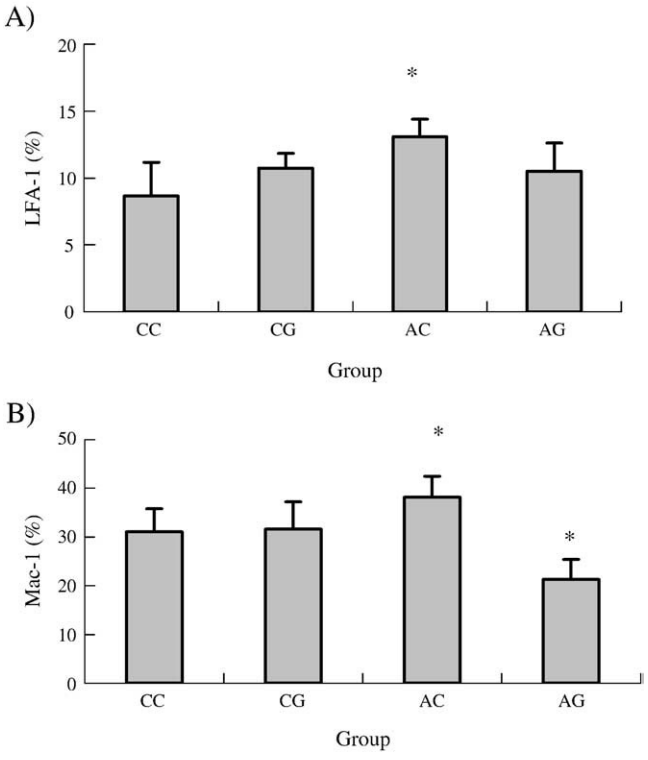

Fig. 1. Blood LFA-1 (A) and Mac-1 (B) expressions as determined by flow cytometry. CC, no arsenic or GLN administered; CG, no arsenic but GLN supplemented; AC, with arsenic but no GLN supplemented; AG, with arsenic and GLN supplemented. Values are means ± SD for 10 mice in each group (n = 10). *$P < .05$ compared with the other 3 groups as determined by ANOVA test.

Table 2
Body weight and plasma GLN concentrations of the groups [a]

	CC	CG	AC	AG
BW (g)	29.7 ± 1.2	28.9 ± 0.8	23.5 ± 1.0*	23.6 ± 0.5*
GLN (nmol/mL)	529.5 ± 54.7	536.3 ± 60.8	410.9 ± 41.3 †	545.0 ± 31.5

Values are expressed as means ± SD for 10 mice in each group (n = 10).

[a] CC, no arsenic or GLN administered; CG, no arsenic but GLN supplemented; AC, with arsenic but no GLN supplemented; AG, with arsenic and GLN supplemented. One-way ANOVA with Fisher test was used to calculate statistical significance.

* Indicates significant difference of $P < .05$ compared with the CC and CG groups.

† $P < .05$ compared with the other 3 groups.

2.4. Statistical analysis

Statistical analyses were performed with SigmaStat version 3.1 software (SYSTAT Software Inc, Chicago, Ill). Data are expressed as the means ± SD. After testing the normality by the bell shape, symmetry of distribution, and kurtosis, all groups performed normal distribution. Differences among groups were analyzed by analysis of variance (ANOVA) with the Fisher least significant difference

Table 3
Interleukin 4, IFN-γ, IL-6, and TNF-α concentrations released by splenocytes after mitogen stimulation for 24 hours

	CC	CG	AC	AG
IL-4	26.2 ± 15.1	8.7 ± 8.4*	28.5 ± 19.8	35.2 ± 19.1
IFN-γ	79.5 ± 31.3	152.2 ± 27.4†	110.0 ± 21.8	139.0 ± 62.5
IL-6	21.0 ± 12.8	8.5 ± 3.5*	25.3 ± 3.1	30.0 ± 16.2
TNF-α	27.2 ± 8.0	25.3 ± 9.2	26.1 ± 5.8	29.8 ± 8.3

Values are expressed as means ± SD for 10 mice in each group (n = 10) and are expressed as picograms per milliliter. The groups are described in the footnote of Table 2. One-way ANOVA with Fisher test was used to calculate statistical significance.

* Indicates significant difference of $P < .05$ compared with the other 3 groups.

† $P < .05$ compared with the CC group.

procedure [21]. A P value less than .05 was considered statistically significant.

3. Results and discussion

There were no differences in initial BWs among the groups. No differences in food and water intake were observed across the groups during the experimental period (data not shown). After feeding the diets for 5 weeks, the BWs of mice in the arsenic groups (AC and AG) were significantly lower (−19.6%) than those of the control groups (CC and CG). Plasma GLN levels in the arsenic group without GLN (AC) were significantly lower (-23%) than those in the control groups. The arsenic group with GLN supplementation (AG) had higher plasma GLN levels than that of AC group and was not different from the control groups (Table 2).

Leukocyte function–associated antigen-1 and Mac-1 expressions on leukocytes were significantly higher in the arsenic group (AC group) than the other groups. There were no differences in LFA-1 expressions between the AG group and the control groups. The Mac-1 expressions in AG group were even lower than the control groups (Fig. 1A and B).

No IL-2 was detectable. Interleukin 4 and IL-6 production by splenocytes in the CG group was significantly lower than the other 3 groups, whereas IFN-γ levels were higher in the control group with GLN supplementation than those without GLN. There were no differences in IL-4, IL-6, IFN-γ, and TNF-α production in the arsenic-exposed groups regardless of whether GLN was supplemented or not (Table 3).

This study showed for the first time that arsenic exposure results in depletion of plasma GLN, and GLN supplementation can normalize plasma GLN levels in mice. In this study, 50 ppm $NaAsO_2$ was fed. This amount of arsenic used was much higher than human exposure to arsenic in drinking water. However, this arsenic content was comparable with studies by others and was shown to accelerate atherosclerosis formation and promote tumor initiation in rodent models [22,23]. The lower BW and plasma GLN levels in the arsenic group indicated that the dose and duration of arsenic administered in this study was not fatal but resulted in metabolic stress in mice.

The metabolism of arsenic plays an important role in its toxicity. In the trivalent state, inorganic and organic arsenic may react with critical thiols in proteins and inhibit their activity [24]. Samikkannu et al [24] showed that arsenite causes oxidative damage to pyruvate dehydrogenase. Pyruvate dehydrogenase catalyzes the reaction that controls the pathway of glycolysis; inactivation of this enzyme may interfere with energy metabolism. Recent studies found that arsenic exposure contributes to metabolic syndrome and diabetes development [25,26]. Moreover, in the human, a potential association between high arsenic exposure and anemia was reported, which could be a marker of malnutrition [27]. Although the biologic mechanism for the high arsenic-associated metabolic disorders are not known, arsenic exposure disturbs overall metabolism in the body and may consequently result in weight loss as observed in this study. The finding that plasma GLN levels were lower in the arsenic group is compatible with previous reports that plasma GLN is reduced during catabolic conditions such as inflammation, infection, and injury [13,16,17].

Leukocyte function–associated antigen-1 is predominantly expressed by lymphocytes, whereas Mac-1 is abundant in polymorphonuclear leukocytes and contributes to neutrophil migration into the site of inflammation [8,28]. Excessive expression of LFA-1 and Mac-1 may induce the inflammatory response and tissue injury [28,29]. Rahimi et al [30] suggested that CD11a, CD11b, and CD18 may be used as indicators for the progression of coronary artery disease. In this study, we found that the arsenic group without GLN had higher LFA-1 and Mac-1 expressions than those in the control groups, whereas GLN supplementation reduced integrin expression in the arsenic group. This finding suggests that GLN supplementation might reduce the interaction between leukocytes and endothelial cells and consequently attenuate the inflammatory response induced by arsenic. An in vitro study performed by our laboratory showed that GLN administration at levels higher than physiologic concentrations reduced neutrophil CD11b expression and the transmigration of neutrophils after stimulation with arsenic [31].

We did not determine plasma cytokines in this study because a previous study reported that plasma IL-1, TNF, and IFN-γ levels are rarely detected in the plasma of injured patients [32]. A previous report by our laboratory also showed that IL-1β, IL-2, and IFN-γ were undetectable even in a septic animal model [33]. Therefore, we analyzed the production of cytokines including IL-2, IL-4, IL-6, IFN-γ, and TNF-α by splenocytes after mitogen stimulation to investigate the effects of GLN supplementation on the systemic immune response under arsenic exposure. Interleukin 6 and TNF-α are proinflammatory cytokines. Interleukin 2 and IFN-γ are produced by T helper type 1 (Th1) lymphocytes. Th1 cytokines enhance cell-mediated immunity. Interleukin 4 is a T helper type 2 (Th2) cytokine

that enhances humoral immunity. The effects of Th1 or Th2 lymphocytes are counter-regulatory [34]. The results showed that GLN supplementation reduced IL-4 and IL-6 production, whereas it enhanced IFN-γ secretion in groups not exposed to arsenic. This finding is partly consistent with a report by Rohde et al [35], which also showed that IFN-γ was enhanced by GLN supplementation. Because there were no differences in cytokine levels between the arsenic-exposed groups regardless of whether GLN was supplemented or not, proinflammatory cytokine modulation might not be responsible for reducing leukocyte integrin expressions. Also, GLN administration did not influence Th1/Th2 cytokine under the present experimental conditions.

A study by Hong et al [36] revealed that GLN-supplemented nutrition protects the liver during hepatic injury by preserving glutathione (GSH) stores. The reduced form of GSH is a major antioxidant and a vital component of a host's defense. The availability of GLN is critical for the generation of GSH stores [37]. We speculate that the antioxidant property of GLN is implicated in reducing arsenic-induced oxidative stress and has a favorable effect on decreasing adhesion molecule expression. Because we did not analyze the GSH levels in this study, whether GLN administration restores GSH levels, improves the antioxidant status of animals, and, thus, decreases leukocyte integrin expression requires further investigation.

In summary, this study showed, for the first time, that arsenic exposure resulted in depletion of plasma GLN. Glutamine supplementation normalized plasma GLN levels and reduced expression of the leukocyte intergins LFA-1 and Mac-1. However, the effects of GLN on in vitro cytokine secretion were not obvious, and cytokine modulation may not be responsible for reducing leukocyte integrin expressions in mice exposed to arsenic.

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