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RESEARCH ARTICLE

Immunological status of White Leghorn chicks hatched from eggs inoculated with ochratoxin A (OTA)

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Abstract

This study was designed to evaluate the immunological status of chicks hatched from the ochratoxin A (OTA)-contaminated eggs. For this purpose, 900 fertile White Leghorn (WL) layer breeder eggs were divided into eight groups (A–H). Group A was maintained as untreated control, whereas Group B was kept as sham control (10 µL of 0.1 M $NaHCO_3$). Groups C, D, E, F, G, and H were injected with 0.01, 0.03, 0.05, 0.10, 0.50, and 1.00 µg OTA/egg, respectively. Eggs were incubated at 37.5°C and 65% relative humidity. Hatched chicks from each group were then maintained separately under standard environmental conditions. At Day 18-of-age, chicks ($n = 10$) from each group were used for lymphoblastogenic response against an intradermal administration of phytohemagglutinin P (PHA-P). At Day 30-of-age, abdominal macrophages, collected from 15 chicks in each group, were utilized for determination of phagocytic potential using sheep red blood cells (SRBC) as particulate antigen and for nitrite production in response to lipopolysaccharide. Antibody (Ab) titers (i.e. total antibodies, IgG, and IgM) against SRBC were determined at 7 and 14 days after primary (at Day 13-of-age) and booster (given 14 days after primary) intravenously administered SRBC doses. The lymphoblastogenic responses of the chicks hatched from OTA-contaminated eggs in response to PHA-P administration were significantly lower at 24, 48, and 72 h after PHA-P injection when compared with responses by control chicks. The percentage of abdominal macrophages displaying phagocytosis of SRBC, the number of SRBC/macrophage, and nitrite production were each significantly lower in cells from chicks in the OTA-administered groups. Total Ab, IgG, and IgM titers against SRBC showed significant reductions in the groups that had been hatched from eggs injected with the higher doses of OTA (as compared with titers associated with chicks in control eggs). These findings suggested that there are substantive immunosuppressive risks in chicks that could be exposed to OTA *in ovo*.

Keywords: Ochratoxin A, fertile eggs, White Leghorn chicks, immune system

Introduction

The widespread occurrence of mycotoxins producing fungi and their ability to grow on the variety of economical important cereal crops has rendered them as unavoidable contaminants of poultry and human food and feeds. The most significant mycotoxins in naturally contaminated poultry feed and feed ingredients are ochratoxins, aflatoxins, T-2 toxin, zearalenone, fumonisins, and deoxynivalenol (Devegowda et al., 1998; Zindine et al., 2006; Binder et al., 2007).

Ochratoxins, secondary metabolites of many species of *Aspergillus* and *Penicillium*, are a group of structurally related compounds well-known for their toxicological effects. Among the ochratoxins, ochratoxin A (OTA) is the most important due to its toxicities (e.g. nephrotoxic, immunosuppressive, hepatotoxic, teratogenic, embryotoxic, and mutagenic) in various avian and mammalian species (Gilani et al., 1978; Muller et al., 1999; Erviti et al., 2005; Elaroussi et al., 2006; Zahoor-ul-Hassan et al., 2010). Further, OTA has also been classified by the International

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Agency for Research on Cancer as a possible human carcinogen (2B) (IARC, 1993).

The intake of mycotoxins-contaminated poultry feed and feed ingredients constitute a major route of exposure for poultry birds (Zinedine et al., 2006; Liu et al., 2007). Besides the dietary intake of mycotoxins, the transmission of these contaminants in the eggs laid by the dams kept on mycotoxins-contaminated diet is another route of exposure for the developing chick embryos, which results in embryonic mortalities, decreased hatchability, and subsequently poor performance of the hatching chicks. In an experiment, Frye and Chu (1977) recovered 2.8 μg OTA/kg in the eggs of hens fed diet contaminated with 5.0 mg OTA/kg body weight (BW) for 2 weeks. In the abdominal yolks and eggs of Japanese quails (*Coturnix coturnix japonica*), Piskorska and Juszkiewicz (1990) detected OTA contents for 4 days after the single oral doses of 1, 5, and 20 mg of OTA/kg BW. Niemiec et al. (1994) detected the OTA contents in the eggs laid by the hens and subsequently in the hatching chicks at Day 1-of-age obtained from the hens fed 2.1 and 4.1 mg OTA/kg feed. The decreases in hatchability percentage and BW gain in chicks hatched from eggs obtained from hens kept on OTA-contaminated diet have been reported by Choudhary et al. (1971). A decrease in weights of immunological organs in the chicks obtained from hens kept on OTA-contaminated diets and decreases in the number of immunoglobulin(s)-bearing cells in the bursa of Fabricius of chicks hatched from OTA-contaminated eggs were reported by Zahoor-ul-Hassan et al. (2011) and Harvey et al. (1987), respectively. Though a lot is known about the immunotoxic effects from direct feeding of OTA to various animal species, information regarding the immunological effects of OTA in chicks hatched from eggs contaminated with OTA are scanty and does not cover effects on cellular/humoral immune responses.

Keeping in view the above-mentioned concerns, the present study was designed to investigate the immunological status of the chicks hatched from the eggs contaminated with different doses of OTA, and to determine the concentration of OTA/egg responsible for the immunosuppression in the hatching chicks, if any.

Materials and methods

Experimental design

A total of 900 fertile White Leghorn (WL) layer breeder eggs were procured from a local breeder farm whose birds were maintained under standard environmental conditions and were routinely vaccinated. Total mycotoxin contents in the eggs (a sample of 10 μL was recovered via syringe from each egg) were analyzed using HPLC (Sizzo and Egmond, 2005). A threshold level of ≥0.02 ng/g was used to differentiate between eggs that would be used in these studies and those that would be discarded.

From all the eggs deemed useful for study, eight groups (i.e. A–H) were established. Group A was to be maintained as an untreated control, and Group B was to be kept as a sham control (to receive 10 μL of 0.1 M $NaHCO_3$). Groups C, D, E, F, G, and H were to be injected with 0.01, 0.03, 0.05, 0.10, 0.50, and 1.00 μg OTA/egg (OTA [CAT #001108, 99.9% purity] from Biopure, Technopark I, Tulln, Austria) dissolved in 0.1 M $NaHCO_3$ (10 μL), using a standard window opening technique described by Vesala et al. (1983). The required amount of OTA was placed onto the air cell membrane and the hole was sealed with melted wax. Eggs were then incubated at 37.5°C and 65% relative humidity.

Chicks hatched from the eggs of the different treatment groups were reared under standard brooding conditions in the Department of Pathology at the University of Agriculture, Faisalabad, Pakistan. Animal rooms were kept at 92°F for the first week and then at 90°F for the remaining period of the study, a 60% relative humidity, and with a 12-h/12-h light–dark cycle; all chicks had access to fresh water and starter rations *ad libitum*. These rations contained 21% crude protein and were verified not to contain OTA or aflatoxin-B_1 (AFB_1) residues at a level >1.0 μg/kg. All studies involving animals were reviewed and approved by the synopsis evolution committee, and experiments were conducted according to the rules and regulations of the Animal Ethics Committee, Faculty of Veterinary Science, at the University of Agriculture, Faisalabad.

Lymphoproliferative response to phytohemagglutinin P

At Day 18-of-age, 10 birds from each group were injected with phytohemagglutinin P (PHA-P) (MP Biomedicals, Inc., Cleveland, OH). For this, 100 μg PHA-P (dissolved in 0.1 mL normal saline) was injected into the intradigital space between digits 3 and 4 of the right foot. At the same time, 0.1 mL normal saline was injected into a similar site in the left foot. Skin thickness was then measured 24, 48, and 72 h post-injection using a constant tension micrometer (Global Sources, Shanghai, China). The thickness response was calculated at each post-injection time point as: cutaneous basophilic hypersensitivity response = (right foot skin thickness – left foot skin thickness) to assess cell-mediated immune response at each time point (Corrier, 1990; Zahoor-ul-Hassan et al., 2011).

Macrophage function assessment

Macrophage functions were assessed following the method of Qureshi and Havenstein (1994), using 15 chicks from each group, as described herein: at Day 30-of-age, a pre-soaked (overnight) 3% suspension of Sephadex G-50 (Sigma, St. Louis, MO) in normal saline was injected intraperitoneally into each chick at a dose rate of 1 mL/100 g BW. At 42 h post-injection, the chicks were euthanized by cervical dislocation and their abdominal cavities flushed with 30 mL heparinized saline (sterile heparin 0.5 U/mL normal saline) in order to permit collection of abdominal exudate cells (AEC). With each harvested AEC sample, after centrifugation at 285×*g* for 10 min, the resultant supernatant was discarded and the

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cell pellet was resuspended in 4 mL RPMI-1640 complete growth medium supplemented with 5% heat-inactivated fetal calf serum and antibiotic–antimycotic solution (each from PPA Laboratories GmbH, Dartmouth, MA). The total number of cells were then counted on a hemocytometer and adjusted to needed levels for use in the experiments outlined below.

Phagocytosis of sheep red blood cells

A 1% suspension of sheep red blood cells (SRBC) in RPMI growth medium was prepared from freshly collected sheep blood (Department of Microbiology, University of Agriculture Faisalabad, Pakistan) for use as particulate antigen for the AEC. Macrophage monolayers were established by the addition of 1×10^6 AEC/mL from each sample into sterile Petri dishes (each dish containing three glass coverslips). After 1 h of incubation in a 5% CO_2/95% air chamber, the coverslips were washed to remove non-adherent cells. The remaining macrophage monolayers were then co-incubated at 41°C in a humidified 5% CO_2 atmosphere for 60 min with a 1.0 mL (3%) suspension of SRBC that was added to each dish. Any non-internalized SRBC were washed away prior to fixing the coverslips in methanol. The coverslips were then stained with Wright–Giemsa stain and mounted onto microscopic slides with DPX mountant (Merck-BDH, Lutterworth, England). A total of 200 macrophages were then scored for phagocytosis and number of SRBC engulfed/macrophage. For this, coverslips were examined under a M7000DB light microscope (Swift, Tokyo, Japan) at 1000× magnification.

Nitrite production

The production of nitrite by abdominal macrophages in response to lipopolysaccharide (LPS, *Escherichia coli* O55:B5, Sigma) was quantified as described by Green et al. (1982). Macrophages (calculated amount) from different groups were cultured in 24-well plates at 41°C in a 5% CO_2 atmosphere. After 1 h of incubation, the non-adherent cells were removed by washing with complete medium and exposed to LPS (1 μg/well) for 24 h. At the end of the incubation period, a aliquot of 200 μL culture supernatant was collected from each well and each mixed with 200 μL Griess reagent (0.1% naphthalenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in 96-well microtiter plates. The plate was incubated at 37°C for 15 min and the optical density (OD) in each well was then measured at 540 nm in a microplate ELISA reader (Biochrom, Holliston, MA). A standard curve for the assay was generated using various dilutions of sodium nitrite solution in RPMI-1640 complete medium. Nitrite levels in the supernatant samples were then extrapolated from a standard curve that was generated in parallel from known concentration sodium nitrite solutions.

Antibody response to SRBC

At Day 13-of-age, a 3% suspension of SRBCs was injected intravenously into 15 birds from each group; a booster dose was injected 14 days later. Blood was collected 7 and 14 days after the primary and booster doses, and the corresponding serum was separated and stored at –20°C until analyzed for antibody response by the methods described by Delhanty and Solomon (1966), Yamamoto and Glick (1982), and Qureshi and Havenstein (1994). In this process, the serum samples were heat-inactivated at 56°C for 30 min and then analyzed for total, mercaptoethanol-2 (ME)-sensitive (IgM), and ME-resistant (IgG) anti-SRBC antibodies. The results were expressed as $\log_2$ of the reciprocal of the last dilution exhibiting agglutination.

Statistical analysis

All data were subjected to analysis of variance tests. Means of the different groups were compared using a Duncan's multiple range test within a MSTATC statistical package (Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI). Data were considered significantly different from one another at a *P*-value ≤0.05.

Results

Lymphoblastogenic response to PHA-P

Lymphoproliferative responses of the chicks, as determined by increased skin thickness at 24, 48, and 72 h after PHA-P injection, are shown in Table 1. After 24 h, chicks in all the groups (except Group B) hatched from OTA-contaminated eggs showed significantly lower skin thickness values as compared with those by chicks from Group A (control group). Values of skin thickness determined at 48 and 72 h after PHA-P injection were significantly lower compared with their control counterpart.

Macrophage functions assays

Abdominal macrophages of chicks hatched from eggs contaminated with OTA displayed significantly lower percentages of phagocytic activity against SRBC as compared with the cells obtained from chicks in control Group A (Table 2). Macrophages from chicks in Group G revealed the lowest percentage (14.53 ± 1.14 [mean ± SD]) of phagocytosis, although those from hosts in Group A had the highest level (27.95 ± 1.57).

Table 1. Lymphoblastogenic response of chicks to intradermal injection of phytohemagglutinin P (PHA-P).

Group (ochratoxin A [OTA] μg/egg)	Skin thickness response (mm)		
	After 24 h	After 48 h	After 72 h
A (0)	0.64 ± 0.02^a	0.34 ± 0.01^a	0.23 ± 0.03^a
B (0.01)	0.63 ± 0.03^a	0.31 ± 0.01^b	0.19 ± 0.02^b
C (0.03)	0.59 ± 0.01^b	0.30 ± 0.02^c	0.18 ± 0.01^c
D (0.05)	0.55 ± 0.03^b	0.29 ± 0.02^d	0.16 ± 0.02^d
E (0.10)	0.49 ± 0.03^c	0.25 ± 0.02^e	0.14 ± 0.01^e
F (0.50)	0.50 ± 0.02^c	0.21 ± 0.01^f	0.11 ± 0.01^f
G (1.00)	0.47 ± 0.02^c	0.17 ± 0.02^g	0.09 ± 0.01^g

In columns, values followed by different letters are significantly different from each other at $P \leq 0.05$.
Values shown are mean ± SD; $n = 10$/group.

The numbers of SRBC/phagocytic macrophage were also seen to be significantly lower in the cells from chicks raised from eggs in each of the OTA experimental groups (except those from Group B) as compared with the cells from the control group chicks. The lowest value (1.22 ± 0.05) was observed with cells from chicks in Group G, and the highest value (2.04 ± 0.16) again was associated with the chicks from Group A. Nitrite production by macrophages challenged with LPS was significantly lower in the cells from chicks in each of the OTA experimental groups (except those from Group B) when compared with cells from the Group A hosts. The highest concentration (24.43 ± 1.39) was observed with Group A (control) chick cells, whereas the lowest value (9.49 ± 0.77) was associated with the cells from chicks in Group G.

Antibody response to SRBC

The antibody (Ab) titers against SRBC in chicks hatched from eggs inoculated with OTA are shown in Table 3. At Day 14 post-primary SRBC injection, total Ab titers in chicks from Groups F and G were significantly lowered as compared with those in control egg chicks. In contrast, the IgG titers in chicks raised from eggs in Groups D, E, F, and G were all significantly lower compared with the values seen in the control birds. There were no statistically significant effects observed among any of the various chicks at Day 7 post-primary SRBC injection.

At Day 7 following the booster SRBC dose, the total Ab titer among chicks from Group G and both IgG and IgM titers in chicks from Groups E, F, and G were significantly lower than the values in the similarly boosted control chicks. At Day 14 post-booster, chicks from Groups F and G showed significantly lowered values of total Ab than their control chick counterparts although the IgG titers from chicks in only Group G were significantly lower relative to values in the control chicks.

Table 2. Macrophage functions of chicks hatched from egg inoculated with ochratoxin A (OTA).

Groups (μg OTA/egg)	% Phagocytic cells	# SRBC/ macrophage	Nitrite production (μM)
A (0)	27.95 ± 1.57^{a}	2.04 ± 0.16^{a}	24.43 ± 1.39^{a}
B (0.01)	24.62 ± 2.12^{b}	1.90 ± 0.15^{a}	20.52 ± 0.91^{ab}
C (0.03)	24.53 ± 1.62^{b}	1.50 ± 0.17^{bc}	16.32 ± 2.60^{bc}
D (0.05)	24.08 ± 1.88^{b}	1.43 ± 0.18^{bc}	19.10 ± 5.41^{b}
E(0.10)	19.95 ± 1.45^{c}	1.58 ± 0.06^{b}	14.18 ± 4.11^{cd}
F (0.50)	18.23 ± 1.52^{c}	1.39 ± 0.19^{bc}	10.40 ± 2.14^{de}
G (1.00)	14.53 ± 1.14^{d}	1.22 ± 0.05^{c}	9.49 ± 0.77^{e}

In columns, values followed by different letters are significantly different from each other at $P \leq 0.05$.
Values shown are mean ± SD; $n = 15$/group.

Discussion

Lymphoblastogenic responses, as determined by increase in skin thickness to intradermal injection of PHA-P, were significantly lower in the chicks hatched from the eggs inoculated with different doses of OTA. Unfortunately, no parallel report is available in the accessible literature about the immunotoxic effects in hatchlings after *in ovo* OTA inoculation. However, the results of acute and chronic ochratoxicosis in the chicks here indicated depression in immunological responses.

Such findings, although potentially specific to the *in ovo* exposure scenario, would be in keeping with those of other investigators who assessed the immunotoxic effects of OTA in several avian and mammalian species. For example, Elaroussi et al. (2006), feeding 400 and 800 ppb OTA for 1–5 weeks to broiler chicks, reported

Table 3. Anti-SRBC antibody titers in chicks from eggs inoculated with ochratoxin A (OTA).

Groups	At Day 7 post-primary injection			At Day 14 post-primary injection		
(μg OTA/egg)	Total Abs	IgG	IgM	Total Abs	IgG	IgM
A (0)	2.40 ± 1.14	1.20 ± 0.45	1.20 ± 1.10	1.80 ± 0.84^{a}	1.60 ± 0.55^{a}	0.20 ± 0.45
B (0.01)	2.40 ± 0.55	1.60 ± 0.55	0.80 ± 0.84	1.60 ± 0.55^{ab}	1.40 ± 0.55^{ab}	0.20 ± 0.45
C (0.03)	3.00 ± 0.01	1.80 ± 0.84	1.20 ± 0.84	1.20 ± 0.45^{ab}	0.70 ± 0.45^{ab}	0.50 ± 0.01
D (0.05)	2.60 ± 0.89	2.00 ± 0.71	0.60 ± 0.55	1.40 ± 0.55^{ab}	1.00 ± 0.01^{b}	0.40 ± 0.55
E (0.10)	2.60 ± 0.89	2.00 ± 1.22	0.60 ± 0.55	1.40 ± 0.55^{ab}	0.50 ± 0.01^{b}	0.50 ± 0.01
F (0.50)	2.80 ± 1.30	1.80 ± 1.30	1.00 ± 0.01	1.00 ± 0.01^{b}	1.00 ± 0.01^{b}	0.40 ± 0.55
G (1.00)	2.00 ± 0.71	1.60 ± 0.89	0.40 ± 0.55	1.00 ± 0.01^{b}	0.60 ± 0.01^{b}	0.40 ± 0.01
Groups	At Day 7 post-booster injection			At Day 14 post-booster injection		
(μg OTA/egg)	Total Abs	IgG	IgM	Total Abs	IgG	IgM
A (0)	6.20 ± 1.48^{a}	5.40 ± 0.55^{a}	1.80 ± 0.84^{a}	4.40 ± 0.55^{a}	3.60 ± 0.55^{a}	0.80 ± 0.84
B (0.01)	5.80 ± 0.84^{ab}	5.00 ± 0.71^{ab}	1.20 ± 0.84^{ab}	3.60 ± 1.14^{ab}	2.80 ± 0.45^{ab}	0.80 ± 0.84
C (0.03)	5.40 ± 0.55^{ab}	4.40 ± 0.89^{abc}	1.20 ± 0.45^{ab}	3.40 ± 0.55^{ab}	3.20 ± 0.45^{ab}	0.20 ± 0.45
D (0.05)	5.60 ± 0.55^{ab}	4.80 ± 0.45^{abc}	1.40 ± 0.55^{ab}	3.60 ± 0.89^{ab}	2.60 ± 0.55^{ab}	0.40 ± 0.89
E (0.10)	6.00 ± 0.71^{ab}	4.20 ± 0.45^{bc}	0.80 ± 0.45^{bc}	3.40 ± 1.14^{ab}	2.80 ± 0.45^{ab}	0.80 ± 0.84
F (0.50)	5.60 ± 0.55^{ab}	4.40 ± 0.55^{bc}	0.80 ± 0.84^{bc}	3.00 ± 0.71^{b}	2.60 ± 0.89^{ab}	0.80 ± 0.45
G (1.00)	4.60 ± 1.52^{b}	3.80 ± 1.10^{c}	0.20 ± 0.01^{c}	2.80 ± 0.84^{b}	2.40 ± 1.34^{b}	0.40 ± 0.55

In columns, values followed by different letters are significantly different from each other at $P \leq 0.05$.
Values shown are mean ± SD; $n = 15$/group.

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significant depressions in the cellular as well as humoral immune responses. Dwivedi and Burns (1985), in young turkeys fed OTA, reported a significant decrease in delayed-type hypersensitivity reactions against intradermal avian tuberculin or bovine serum albumin (BSA). Muller et al. (1999) reported a similar decrease in the immune response in weanlings fed OTA; specifically, their findings reported on a decrease in skin thickness in response to PHA injection and decreases in the phagocytic activities of polymorphonuclear cells. The decrease in the skin thickness responses in the chicks here may be a result of decreases in the population and/or impaired function of local and circulating immune cells (Al-Anati and Petzinger, 2006) in these hosts. Alvarez et al. (2004) reported suppressed activity of cytotoxic T-lymphocytes in Wistar rats administered 50 μg OTA/kg BW. In mice, in response to OTA administered at 2600 μg/kg feed, significant decreases in the numbers of T-lymphocytes subpopulations, that is, $CD4^+$ and $CD8^+$, and decreases in responses of thymocytes and splenocytes to concanavalin A were reported by Thuvander et al. (1995).

Abdominal macrophages harvested from the chicks, hatched from the eggs inoculated with OTA, showed significant decrease in their activity in term of their phagocytic potential to SRBC and also significant decrease in the concentration of nitrite production when stimulated by LPS. Although, not in the progeny of OTA fed dams, the decrease activity of macrophages during ochratoxicosis has been reported by many researchers in different animal models. Alvarez et al. (2004) reported significant lower bacteriolytic capability of macrophages collected in the Wistar rats administered 50 and 450 μg OTA/kg BW. Similar decrease in the phagocytic potential of heterophils was reported during ochratoxicosis in the broiler chicks by Chang and Hamilton (1980). This noted decrease in the phagocytic potential of heterophils may be due to the toxicological potential of OTA on immunological organs and subsequently on immunological cells during embryonic development.

The depression in humoral immune response of the hatched chicks in the present study was depicted by decreased antibodies titers against intravenous administration of SRBC. This depression in the antibody titers may be due to damage caused by OTA in bursa of Fabricius of chicken embryos, which ultimately resulted in the decrease in the number of immunoglobulins-producing cells as was reported by Harvey et al. (1987) in OTA inoculated chicken embryos. This hypothesis may be further strengthened by the results of a study conducted by Santin et al. (2002) in broiler chicks fed OTA at 2 ppm, showing decrease in the mitotic figures in bursa of Fabricius and subsequently depressed humoral immune response, and Moura et al. (2004) showed the decrease in the number of lymphocytes in the broiler chicks given intraperitoneal injection of OTA at 0.04 mg/ kg BW. The decrease in antibody production to SRBCs in the Balb/c mice exposed to OTA at 250 and 2600 μg/kg diet and in mice administered 0.005 μg OTA/ kg BW have been reported by Thuvander et al. (1995) and Haubeck et al. (1981), respectively. From the results of present study, it can be concluded that inoculation of OTA into the fertile eggs not only resulted in embryonic mortalities and decreases in hatchability, but also ultimately gave rise to a suppression of immunological responses of the hatching chicks.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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