

# IMMUNOLOGY

## Immune Responses in Chickens Against Lipopolysaccharide of *Escherichia coli* and *Salmonella typhimurium*

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**ABSTRACT** Immunization of chickens with whole bacteria results in the production of antibodies specific to lipopolysaccharide (LPS), a major constituent of the outer membrane of Gram-negative bacteria. However, there is relatively limited information available concerning immune response of purified LPS in this species. In the present study, immune responses were examined in serum and egg yolk from two groups of chickens injected with entire LPS from *Escherichia coli* and lipid A

free LPS from *Salmonella typhimurium*. The results demonstrated that the increase of antibody activity occurs first in serum, and then in egg yolk with a lag in time of 1 to 3 wk in both groups of chickens. However, the time of elevated levels of antibody activity was much shorter in chickens immunized with *S. typhimurium* LPS (< 1 wk) than in those immunized with *E. coli* LPS (4 wk). A lack of lipid A in the *S. typhimurium* antigen may be a factor related to this difference.

(Key words: chicken, lipopolysaccharide, egg yolk antibody, *Escherichia coli*, *Salmonella typhimurium*)

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### INTRODUCTION

Endotoxic lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*, which are known as the agents causing gastroenteritidis in humans (Morrison and Ryan, 1987). The LPS consists of a core oligosaccharide, O-specific polysaccharide, and lipid A, which is a major toxin found in the bacteria (Raetz, 1990).

In chickens, serum IgG is accumulated in the egg yolk by the active transport during oogenesis (Rose and Orlans, 1981). Other antibody isotypes, IgA and IgM, are also transferred to the egg. They are, however, found in the egg white but not in the egg yolk. Thus, the IgG in the egg yolk is often termed IgY (Leslie and Clem, 1969). The concentration of IgG in the yolk is higher than in the serum (Rose *et al.*, 1974; Larsson *et al.*, 1993).

Production of IgG in hen's egg yolk is an efficient economical method to raise polyclonal antibodies, in that bleeding of birds is not necessary and purification of IgG is relatively simple (Akita and Nakai, 1992). Chickens have been immunized with several antigens. These included bovine serum albumin (Ermeling *et al.*, 1992), human serum albumin (Rose and Orlans, 1981; Löscher *et al.*, 1986),  $\alpha$ -subunit of insulin receptor (Song *et*

*al.*, 1985), viruses (Polson and Wechmar, 1980; Bar-Joseph and Malkinson, 1980; Gardner and Kaye, 1982) and bacteria (Shimizu *et al.*, 1988). Immunization of chickens with formalin-treated cells of *E. coli* leads to the production of antibodies specific to LPS (Shimizu *et al.*, 1988). However, there is relatively limited information available concerning immune responses of chickens against purified LPS. The present study was undertaken to investigate immune responses of chickens against two antigens, LPS (entire LPS) from *E. coli* and lipid-free polysaccharide (detoxified LPS) from *S. typhimurium*. These antigens were commercially available products with high purity.

### MATERIALS AND METHODS

#### Lipopolysaccharide

Lyophilized samples of LPS from *E. coli* and *S. typhimurium* were obtained from Sigma Chemical Co.<sup>2</sup> and used as antigens. The LPS from *E. coli* was composed of O-specific polysaccharide, a core oligosaccharide, and a lipid A (entire LPS), whereas the LPS from *S. typhimurium* lacked lipid A (detoxified LPS) (supplier's information).

#### Immunization of Hens

All chickens were cared for in accordance with the Canadian Council on Animal Care guidelines of animal welfare. Immunization of hens was carried out as described (Ivanyi and Czerny, 1969). Twelve 40-wk-old

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<sup>2</sup>Sigma Chemical Co., St. Louis, MO 63178-9916.

TABLE 1. Protein and IgG concentrations in egg yolk and water soluble fraction (WSF)<sup>1</sup>

Lipopolysaccharide source	Fraction	Protein	IgG	IgG:Protein
		(mg/mL)		
<i>Escherichia coli</i>	Egg yolk <sup>2</sup>	119.2 ± 22.2	10.5 ± 3.3	0.09
	WSF	31.9 ± 5.5	8.6 ± 2.9	0.27
	Percentage recovered in WSF	26.8	81.9	
<i>Salmonella typhimurium</i>	Egg yolk <sup>3</sup>	131.5 ± 20.7	11.9 ± 3.4	0.09
	WSF	33.7 ± 6.6	9.3 ± 2.8	0.28
	Percentage recovered in WSF	25.6	78.2	

<sup>1</sup>Eggs were collected during the immunization period of 35 to 55 d.

<sup>2</sup>Twenty-eight egg yolks were analyzed.

<sup>3</sup>Twenty-one egg yolks were analyzed.

White Leghorn hens were divided into two groups. The average body weights of birds to be immunized with *E. coli* and *S. typhimurium* antigens were 1.85 and 1.81 kg, respectively. Each antigen (400 µg/mL of PBS, pH 7.2) was emulsified with an equal volume of Freund's complete adjuvant.<sup>3</sup> Each hen was injected i.m. at four different sites (0.25 mL per site) of breast muscles (two sites per left or right breast muscle). Booster shots were given i.m. 2 wk after the first injection with the same dose emulsified with Freund's incomplete adjuvant.<sup>3</sup> Blood samples were collected from the wing vein on 3, 8, 14, 21, 28, 42, and 56 d after the initial injection. Eggs were collected every day and stored at 4 C until used.

### Separation of Antibodies from Egg Yolk

A water-soluble extract fraction (WSF) that contained immunoglobulins was prepared from egg yolk by the method of Akita and Nakai (1992). The egg yolk was physically separated from the egg white and poured into a graduated cylinder by puncturing yolk membrane. The egg yolk was diluted with 6 vol of distilled water (acidified with 0.1 M HCl to give pH 5.0) and the mixture was incubated at 4 C for 6 h. After centrifugation at 10,000 × g and 4 C for 25 min, the WSF was filtered through Whatman No. 1 filter paper in the cold and was assayed for protein and IgG. The anti-LPS antibody activity in WSF was determined by ELISA.

### ELISA

Serum and egg yolk antibodies raised against LPS antigens were assayed by an ELISA procedure. Microtitre plates (Dynatech Immunolon III)<sup>4</sup> were used as a solid support and were coated with LPS antigens. A 10 µg/mL concentration of antigen in carbonate-bicarbonate buffer

(0.05 M, pH 9.6) was added to each well and incubated for 24 h at room temperature. The plates were washed three times with deionized water. After washing, 150 µL of 1% (wt/vol) solution of BSA in carbonate-bicarbonate buffer (0.05 M, pH 9.6) was added to each well, and incubated at 37 C for 30 min. The BSA solution was then discarded and each well was washed three times with PBS-Tween 20 (0.05%) (PBS-Tw). Diluted serum and WSF were added to the plate and incubated at 37 C for 1 h. The plates were washed three times with PBS-Tw and 150 µL of rabbit anti-chicken IgG conjugated with horseradish peroxidase<sup>2</sup> (1:1,000 in PBS-Tw) was added to each well. After incubation at 37 C for 1 h, plates were washed three times with PBS-Tw, followed by addition of 100 µL of freshly prepared substrate solution, 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in 0.05 M phosphate citrate buffer (pH 5.0) containing 0.03% sodium perborate. The reaction was continued for 30 min. Each plate had control wells containing sera and WSF from unimmunized chickens. Absorbance of chromophore produced in the reaction mixture was read at 405 nm using a Bio-Tek EL 309 microplate reader.<sup>5</sup>

### Immunodiffusion

Radial immunodiffusion (RID) was performed using modifications of methods described by Mancini (1965). Solution A was prepared by mixing 0.3 mL rabbit anti-chicken IgG with 1.7 mL barbital buffer (50 mM sodium barbital, 10 mM barbital, pH 8.6) and incubating in a 56 C water bath. Solution B was prepared by mixing 70 mg of agarose with 4.6 mL barbital buffer and 0.4 mL of 0.35% (wt/vol) sodium azide, and holding the mixture in a boiling water bath until the agarose dissolved. Solutions A and B were then equilibrated at 56 C, mixed well, and poured into RID plates. Serum and WSF (both 6 mL) and IgG standards (6 mL) containing 0.05 to 0.8 mg of chicken IgG were added to 2.5 mm diameter wells. A standard curve was obtained by plotting square values of diameter of the precipitation rings developed at room temperature for 2 to 3 d. The IgG concentrations of serum and WSF were determined by reference to this curve. No correction was made for the recovery of IgG (Table 1) during isolation of WSF.

<sup>3</sup>Difco Laboratories, Detroit, MI 48232.

<sup>4</sup>Dynatek Labs, Alexandria, VA 22314.

<sup>5</sup>Bio-Tek Instruments, Inc., Winooski, VT 05404.

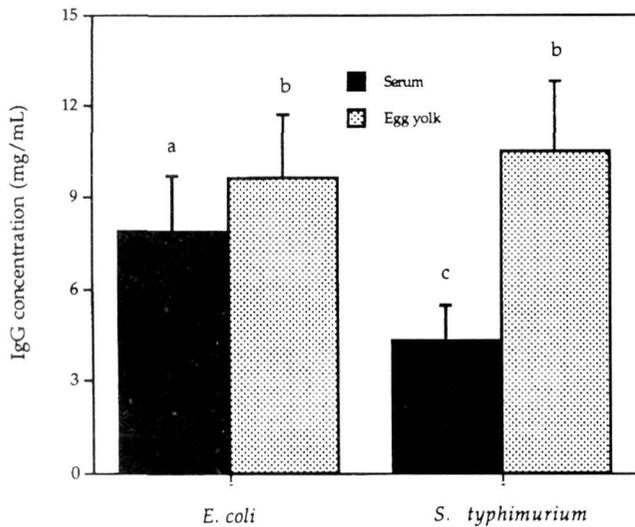


FIGURE 1. The IgG concentrations in serum and egg yolk from chickens immunized with lipopolysaccharides of *Escherichia coli* and *Salmonella typhimurium*. Each bar indicates mean  $\pm$  standard deviation based on twenty-four observations. Different letters represent significant differences ( $P < 0.05$ ) between sources of antibody or treatment groups.

### Protein Determination

Protein concentrations in serum and WSF were determined by the Bradford method (Peterson, 1983) using BSA as the reference protein.

### Statistical Analysis

A *t* test for the paired two samples ( $P < 0.05$ ) was used to evaluate the difference between means.

## RESULTS AND DISCUSSION

Concentrations of IgG determined in either serum or egg yolk were relatively constant ( $P > 0.05$ ) during the immunization period in both groups of hens. A similar trend has been reported previously in egg yolk IgG during immunization of chickens (Shimizu *et al.*, 1988). Thus, analytical values obtained in different days were pooled within a group (Figure 1). In both groups, concentrations of IgG were lower ( $P < 0.05$ ) in serum (average 6 mg/mL) than in egg yolk (10 mg/mL). A similar trend has been reported previously (Rose *et al.*, 1974). It appears that IgG secreted into the hen's circulatory system was selectively accumulated in the egg follicle. The IgG values were similar between the two groups in egg yolks, but lower ( $P < 0.05$ ) in the group immunized with *S. typhimurium* antigen in serum. This difference is difficult to explain. Previous reports showed that serum IgG levels in chickens are near 6 mg/mL (Rose *et al.*, 1974; Yokoyama *et al.*, 1992). The value was similar to the mean IgG value obtained in this study (see above).

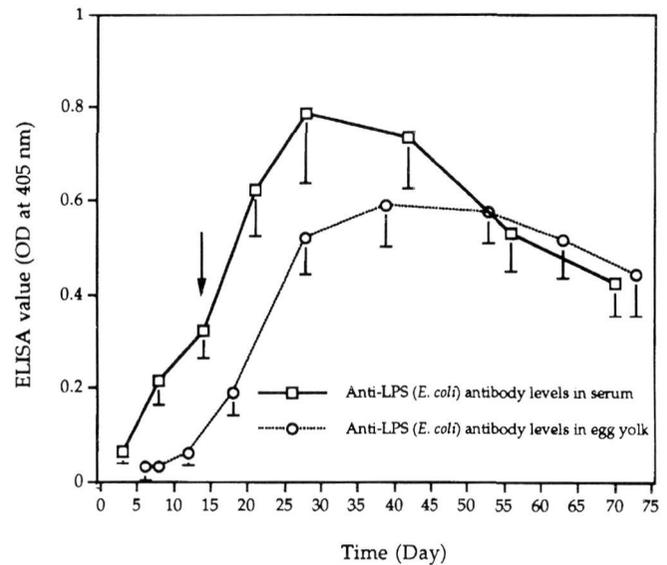


FIGURE 2. Changes of antibody activity in chicken serum and egg yolk during the immunization period. Anti-lipopolysaccharide (LPS) (*Escherichia coli*) antibody activities in serum and egg yolk were measured by ELISA using LPS as antigen and are expressed as ELISA absorbances at 405 nm for serum and egg yolk at 1:1,000 dilution. Means are of triplicate-samples but from one or multiple eggs. Vertical bars indicate the standard deviation. Arrow indicates the time of booster injection of LPS from *E. coli*.

Analytical data of yolk and its WSF from eggs collected during the 35 to 55 d immunization period are shown in Table 1. Protein and IgG concentrations in yolk were similar ( $P > 0.05$ ) between the two groups, and averaged 125.4 and 11.2 mg/mL, respectively. These values are closer to those reported by Cook and Briggs (1977) (160 mg protein/mL) and Akita and Nakai (1992) (12 mg IgG/mL). An average of 26% of the protein and 80% of the IgG were recovered in WSF, in which the ratio of IgG to protein was three times greater ( $P < 0.05$ ) than in egg yolks.

The immune response of laying hens against the LPS antigens were monitored by measuring antibody activities in serum and egg yolk by ELISA. In serum the level of activity of anti-*E. coli* LPS (Figure 2) sharply increased ( $P < 0.05$ ) after the initial immunization, became highest at 28 d ( $P < 0.05$ ). The level was maintained up to 42 d and decreased ( $P < 0.05$ ) thereafter. On the other hand, the level of antibody activity in yolk (Figure 2) started to increase ( $P < 0.05$ ) 13 d after the initial immunization. The lag in time between serum and egg yolk was approximately 1 wk. The antibody activity became highest at 38 d ( $P < 0.05$ ), and remained relatively constant up to 63 d. This suggests that the elevated activity of anti-LPS IgG can be maintained much longer in the yolk (approximately 4 wk) than in the serum (< 1 wk). Similarly, Shimizu *et al.* (1988) in a study of immunized hens reported that the maximum level of anti-LPS antibody activity in the yolk persisted longer than that in the serum.

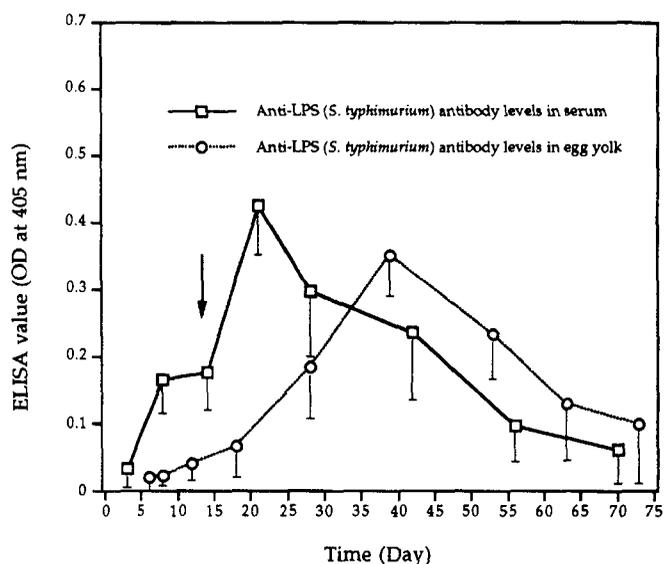


FIGURE 3. Changes of antibody activity in chicken serum and egg yolk during the immunization period. Anti-lipopolysaccharide (LPS) (*Salmonella typhimurium*) antibody activities in serum and egg yolk were measured by ELISA using LPS as an antigen and are expressed as ELISA absorbances at 405 nm for serum and egg yolk at 1:1,000 dilution. Means are of triplicate-samples but from one or multiple eggs. Vertical bars indicate the standard deviation. Arrow indicates the time of booster injection of LPS from *S. typhimurium*.

In hens immunized with *S. typhimurium* antigen, the highest anti-LPS antibody activity was found on 21 and 39 d in serum and yolk, respectively (Figure 3). The activity decreased rapidly ( $P < 0.05$ ) thereafter. Thus, the period of elevated antibody activity was much shorter in chickens immunized with *S. typhimurium* antigen than in those immunized with *E. coli* antigen.

Shimizu *et al.* (1988) immunized chickens with whole cells of *E. coli*, and observed elevated levels of anti-LPS activity, suggesting that LPS is a potent antigen in chickens. The results obtained in the present study were consistent in this regard. However, the results showed different immune responses between the two antigens, in that the time of elevated antibody activity was much longer in hens immunized with *E. coli* LPS than in those immunized with *S. typhimurium* LPS. Whether or not this is related to the lack of lipid A in the latter is unknown. However, if so, the entire LPS is advantageous over the lipid-free LPS for the production of anti-LPS antibody in chickens.

In conclusion, immune responses of chickens were studied with two antigens, entire LPS from *E. coli* and lipid-free LPS from *S. typhimurium*. The latter was found to be related to the shorter time of elevated antibody activity than the former.

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