

Immunogenicity of Ad Libitum Drinking Water Administration of Bovine Serum Albumin in Leghorn Chickens

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ABSTRACT Oral administration of protein antigen in solution routinely leads to development of oral tolerance in most mammals but has been reported to be fully immunogenic in chickens. Previous studies, including several performed by our laboratory, have demonstrated that oral administration of discrete amounts of BSA for 6 consecutive days is fully immunogenic. This study was performed to determine immunoresponsiveness to protein antigen administered ad libitum at low levels in drinking water compared with i.p. and oral gavage routes of administration. Seven days following the last oral immunization, serum was assayed for IgG, bile for IgA, and tissue culture supernatant from 3 distinct lower intestinal regions for IgG and IgA in immunized and nonimmunized single-comb White Leghorn chickens. Systemic responses in the serum of experimental birds revealed a

greater ($P < 0.001$) IgG response when BSA was administered via i.p. injection or by drinking water compared with gavage administration or nonimmunized controls. Responses measured in bile revealed that BSA administration in the drinking water resulted in a greater ($P < 0.001$) secretory IgA response compared with i.p. or gavage administration, and negative control groups. Intestinal antigen specific IgG, but not IgA, was elevated ($P < 0.05$) in all intestinal areas tested in birds immunized against BSA by drinking water and i.p. routes of administration, compared with other experimental groups. Taken together, the present experiments demonstrate that ad libitum drinking water administration of a protein antigen is as effective as i.p. administration or gavage routes of antigen exposure and potentially describe a novel approach to immunization of commercial poultry with purified protein antigens.

(Key words: antibody response, BSA, drinking water, intestine, serum)

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INTRODUCTION

Oral administration of soluble, nonreplicating protein antigen has been reported to lead to oral tolerance in many mammals, resulting in transitory induction of secretory IgA (sIgA) and suppression of systemic IgG. Other mammals, such as the guinea pig, are anaphylactically sensitized by administration of oral antigen (Challacombe and Tomasi, 1980; Miller and Cook, 1994). In chickens, however, protein antigen administered orally to chicks older than 10 d of age induces a strong humoral response involving elevated serum levels of IgG and IgA isotypes and does not sensitize them to delayed type hypersensitivity after repeated antigen exposure (Miller and Cook, 1994; Klipper et al., 2001). Because resistance to some pathogens correlates with sIgA secretion at mucosal sites, rather than systemic IgG or IgM circulating antibody lev-

els, this distinction between chicken and mammalian models may have important implications for subunit vaccine delivery in commercial poultry production (Tomasi and Bienenstock, 1968; Davis et al., 1978).

Previous studies have determined that a number of factors influence the response to orally administered protein antigen in chickens. These factors include the form in which it is given, the concentration of antigen, the time at which it is administered, and the route of administration. When BSA was administered in a powdered form to chicks older than 10 d of age, it neither induced a systemic immune response nor increased the response to subsequent immunizations. This is in direct contrast to the responses observed in similar animals to dissolved BSA fed orally, which included a systemic antibody response. However, chicks younger than 8 to 10 d of age are not responsive to orally administered dissolved BSA and develop tolerance to it depending upon the initial dose (Klipper et al., 2001). This responsiveness to antigen correlates with the colonization of the gut mucosa with

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Abbreviation Key: HBSS = Hank's balanced salt solution.

B and T lymphocytes and the subsequent cytokine expression and production that is necessary for regulating humoral responses at mucosal sites (Bar-Shira et al., 2003). The route of administration has also been demonstrated to be a factor. Three administrations 1 wk apart of BSA via oral gavage was shown to induce low and inconsistent IgG levels without adjuvant (Hoshi et al., 1998, 1999), whereas the same solution fed in trickle fashion via syringe resulted in a strong and consistent serum IgG response. When dosage or concentration of this type of antigen administration was investigated for optimal responsiveness, it was determined that as little as 2 mg fed for 6 consecutive days resulted in a strong and consistent serum IgG response (Klipper et al., 2000).

Given the evidence of robust humoral responses elicited to orally administered soluble BSA and the fact that tolerance seems to be an age-dependent event in chickens, oral administration of protein antigen may be practical in a commercial setting for mass vaccination. For this approach to vaccination to be practical, mass immunization strategies must be investigated. The most efficient method for oral administration would be via drinking water. The purpose of this study was to evaluate the efficacy of low dose, ad libitum administration of a protein antigen in the drinking water to immunologically mature chickens in eliciting systemic and local intestinal antibody responses. This route of administration was compared with oral gavage and i.p. routes of administration.

MATERIALS and METHODS

Experimental Birds

Nonvaccinated, male single-comb White Leghorn chicks² were obtained from a local commercial hatchery on day of hatch and placed in floor pens at an initial density of 18 birds/m² with pine shavings as litter. They were maintained at age-appropriate temperature and fed a corn-soy chick starter ration, lacking animal fat or protein, formulated at or above NRC recommendations (National Research Council, 1994) for poultry. Feed and water were provided ad libitum.

Experimental Design and Immunization

In experiments 1 and 2, day of hatch chicks were divided into 5 experimental groups: negative control, i.p. alum control, i.p. BSA, BSA gavage, and BSA in drinking water. To immunize chickens to BSA by i.p. administration, 2 successive i.p. injections consisting of 5 μ g BSA adsorbed to 5 mg alum (AlK(SO₄)₂) by sodium hydroxide precipitation were administered to chickens on d 12 and 18 of each experiment. Nonimmunized alum control chickens received i.p. injections of 5 mg of alum only on d 12 and 18 of each experiment. Chickens in the gavage

group received a per os gavage once daily with 12.5 mg of BSA in 0.5 mL of water on d 12 through 17 of each experiment. Chickens in the drinking water group were given ad libitum access to a bell drinker with water containing 1.4 mg/mL of BSA on d 12 through 17 of each experiment.

Sampling

On d 25 of each experiment, that is, 7 d following the final i.p. immunization and 8 d following the last oral administration, 1 mL of blood was drawn from the wing (bicipital) vein and allowed to clot for serum collection. Serum was transferred to a clean microcentrifuge tube and was frozen at -20°C until assayed by indirect ELISA. The chickens were then euthanized, and, in experiment 2 only, intestinal sections were collected to assay mucosal antibody production stimulated by the evaluated routes of immunization.

Ex Vivo Culture of Intestinal Tissue

To measure antigen-specific IgG or IgA production by intestinal sections following immunization (experiment 2), we adopted the method described by Zigterman et al. (1993) with slight modification. Immediately following euthanasia, intestinal tissue corresponding to the distal ileum, cecal tonsils, and the distal cecum were removed and immersed in ice cold Hank's Balanced Salt Solution (HBSS) containing 500 IU/mL of penicillin and 500 μ g/mL of streptomycin. Gut contents were removed by gently flushing each tissue section with HBSS. Tissues were then cut into pieces weighing 0.3 g each, and each piece was then cut into 3 smaller pieces and washed with HBSS containing penicillin and streptomycin. Tissues, respective for each bird sampled within each experimental group, were then suspended in 6 mL of RPMI 1640 supplemented with 100 IU/mL of penicillin, 100 μ g/mL of streptomycin, 100 μ g/mL of gentamicin, and 40 mM HEPES buffer. Suspensions were centrifuged (5 min, 300 \times g), and 800- μ L aliquots of supernatant (ileum, cecal tonsil, or distal cecum supernatant; labeled t = 0) were taken.

The tissues were resuspended in the remaining medium and incubated at 41°C, 5% CO₂, and 95% air in 12-well culture plates for 16 h. After 16 h in culture, aliquots were removed and labeled as t = 16 for each respective sample. Antigen-specific IgG and IgA levels measured during ex vivo intestinal culture for each experimental bird were determined using indirect ELISA by subtracting the t = 0 values from t = 16 values, to measure antibodies produced only during the 16-h incubation.

Absorption of Cross-Reactive Antibodies and ELISA

Serum or collected media from intestinal tissue culture, respective for each experimental bird evaluated, was preincubated overnight at 4°C with 40 mg of chicken albumin

²W-36 Line, Hy-Line International, West Des Moines, IA.

per mL and diluted to 1:320, 1:640, and 1:1,280 for serum samples and 1:5, 1:10, and 1:20 for intestinal media samples to eliminate potential cross-reactive antibodies. Indirect ELISA was performed on all serum or intestinal tissue culture samples using 96-well plates coated with 5 μ g of BSA per well. Following overnight incubation, plates were rinsed with PBS-Tween (pH 7.4, 0.5% Tween 20), 150 μ L of serum or media was added, and plates were agitated for an additional 2 h. Plates were rinsed, horseradish peroxidase-conjugated goat antichickens IgG or IgA³ was added to each well, and plates were agitated for 1 h. Plates were rinsed, and a substrate solution containing 100 μ L of dimethyl sulfoxide with 1 mg of tetramethylbenzidine in 10 mL of sodium acetate buffer (pH 5.5) was added.

After 15 min the reaction was stopped by adding 50 μ L of 1 M sulfuric acid. Absorbance was read by a multiwell plate reader at a wavelength of 450 nm.

Statistical Analysis

Samples were read in duplicate and normalized for plate effects by dividing by a positive standard. The mean absorbance of samples was log transformed to normalize variance and analyzed using the univariate GLM for ANOVA.

Statistically different means ($P < 0.05$) were further separated using Duncan's multiple range test. All analyses were performed using SPSS software (SPSS, 2001).

RESULTS AND DISCUSSION

It has been well established that oral administration of protein in solution is immunogenic and not tolerogenic in domestic chickens (Miller and Cook, 1994; Klipper et al., 2000). This has been demonstrated by feeding discrete amounts of soluble antigen to chickens of an immunocompetent age for 6 to 14 consecutive days (Miller and Cook, 1994; Klipper et al., 2000), as evidenced by specific antibody production in serum and bile. Alternative approaches of antigen administration, such as once weekly feedings, do not induce a humoral response in the absence of adjuvant (Hoshi et al., 1998, 1999).

In the present study, serum IgG responses measured in experiments 1 and 2 are shown in Tables 1 and 2, respectively. Although the background absorbance varied between experiments, trends and observed significance between experimental groups in both experiments were the same. Bovine serum albumin administered ad libitum in the drinking water resulted in the generation of greater antibody levels ($P < 0.001$) when compared with levels in chickens in negative control and oral gavage groups. Similarly, IgG levels in chickens in the i.p. BSA group were greater ($P < 0.001$) than all experimental groups except the drinking water BSA group, indicating

TABLE 1. Antigen-specific serum IgG and bile IgA responses¹ in chickens immunized with BSA by intraperitoneal or oral routes of administration (experiment 1)

Group	n	Serum IgG	Bile IgA
Control	4	0.07 \pm 0.10 ^b	0.68 \pm 0.16 ^b
BSA in drinking water	10	0.47 \pm 0.07 ^a	1.35 \pm 0.10 ^a
BSA – oral gavage	9	0.15 \pm 0.07 ^b	0.41 \pm 0.11 ^b
Control (alum) – i.p. injection	3	0.02 \pm 0.12 ^b	0.74 \pm 0.18 ^b
BSA – i.p. injection	9	0.59 \pm 0.07 ^a	0.89 \pm 0.11 ^b

^{a,b}Means \pm SE within columns with no common superscript differ significantly at the following level of significance: $P < 0.001$.

¹Data represent mean \pm SE of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, bile samples were diluted 1:80, and media from intestinal culture was diluted 1:10.

that oral drinking water antigen administration was as efficacious as i.p. administration with adjuvant.

Biliary IgA responses, as shown in Tables 1 and 2, revealed a different trend. Although the background was high in both experiments, BSA administered to chickens via drinking water induced higher ($P < 0.001$) levels of sIgA than i.p., gavage, or either of the control groups. Intestinal IgG responses (Table 2) were similar to responses measured in the serum. In the distal ileum, cecal tonsils, and cecum, IgG levels were higher ($P < 0.05$) in chickens in the i.p. BSA and drinking water experimental groups than those observed in the oral gavage and control groups. Intestinal sIgA responses, however, did not differ significantly between the groups in any of the intestinal regions evaluated (data not shown).

These data confirm that oral administration of soluble BSA induces serum and bile antibody responses and also establishes that a local intestinal antibody response is measurable 7 d following the last time of immunization, indicating that specific antibodies were produced by lymphocytes in the distal ileum, cecal tonsils, and distal cecum in response to oral BSA immunization. Although measured responses consisted of primarily IgG secretion, it bears mentioning that only one time point was evaluated during this assessment of intestinal antibody secretion. That sIgA levels were observed not to differ at 7 d after the last immunization, whereas IgG secretions did, is not a unique observation. Other studies have reported similar findings in response to challenge with *Eimeria* species or a purified recombinant antigen from *E. tenella* (Girard et al., 1997, 1999). It is likely that if measured on a later day, IgA production may have been elevated in the intestinal sections tested, as seen in the studies previously mentioned.

As stated above, ad libitum oral administration of soluble protein antigen elicits an immune response in commercial lines of single-comb White Leghorn chickens. Although this study was limited to single-comb White Leghorn chickens, other studies have reported a similar responsiveness to oral BSA administration in broiler chickens (Klipper et al., 2000). When compared with negative controls and crop-gavaged chickens, birds receiving BSA by drinking water administration produced higher levels of antigen-specific antibodies. It is noteworthy that

³Bethyl Laboratories, Montgomery, TX.

TABLE 2. Antigen-specific serum IgG, intestinal IgG, and bile IgA responses¹ in chickens immunized with BSA by intraperitoneal or oral routes of administration (experiment 2)

Group	n	Serum IgG	Ileum IgG	Cecal tonsils IgG	Distal ceca IgG	Bile IgA
Control	5	0.16 ± 0.08 ^B	0.02 ± 0.01 ^b	0.03 ± 0.01 ^b	0.03 ± 0.01 ^b	0.73 ± 0.14 ^B
BSA in drinking water	5	0.55 ± 0.07 ^A	0.13 ± 0.03 ^a	0.12 ± 0.03 ^a	0.12 ± 0.03 ^a	1.11 ± 0.09 ^A
BSA – oral gavage	5	0.20 ± 0.07 ^B	0.03 ± 0.01 ^b	0.05 ± 0.02 ^b	0.05 ± 0.02 ^b	0.42 ± 0.09 ^B
Control (alum) – i.p. injection	5	0.14 ± 0.08 ^B	0.01 ± 0.01 ^b	0.01 ± 0.01 ^b	0.01 ± 0.01 ^b	0.43 ± 0.16 ^B
BSA – i.p. injection	5	0.45 ± 0.07 ^A	0.18 ± 0.10 ^a	0.12 ± 0.04 ^a	0.12 ± 0.04 ^a	0.69 ± 0.09 ^B

^{a,A,B}Means ± SE within columns with no common superscript differ significantly at the following levels of significance: ^a $P < 0.05$; ^A $P < 0.001$.

¹Data represent mean ± SE of peak absorbance values determined by indirect ELISA. Serum samples were diluted 1:640, bile samples were diluted 1:80, and media from intestinal culture were diluted 1:10.

IgG levels in those chickens were not significantly different than levels in chickens given 2 i.p. injections of BSA with alum as an adjuvant.

Observations of drinking water administration being more efficacious than powdered forms of administration in eliciting humoral immune responsiveness were also reported by Klipper and coworkers (2001). One possible explanation may involve crop distension associated with the single bolus per os administration. Crop distension, in this scenario, may cause the release of proteolytic pepsinogen and HCl by the proventriculus, which might result in degradation of the protein before adequate amounts could reach lymphoid tissue at distal sites in the intestine. This hypothesis is supported by the findings of Klipper et al. (2000), who observed that feeding powdered forms of BSA to mature chicks induced neither an immune response nor tolerance, and by the results of Hoshi et al. (1998), who demonstrated that gavage administration did not induce immunity and may induce tolerance.

Regardless of the mechanisms involved in the observed responsiveness to oral antigen in the chicken, the present investigation confirms that soluble oral protein administration induces systemic and local humoral immune responses. These findings suggest that subunit protein vaccines, administered by a drinking water route of delivery, may be a feasible option for commercial productions or smaller breeder flocks. Although further investigation into this route of mass vaccination is clearly needed to support such a claim, the ease of administration to large numbers of birds by this approach represents a distinct advantage, even over the current approach of oral administration of many live and attenuated vaccines for enteric pathogens. Additional research should include investigation into variability in immunogenicity of antigens isolated from specific pathogens, as well as the level of protection induced by these antigens. Furthermore, the duration of immunity elicited by oral protein antigen administration with and without adjuvant needs to be studied.

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