IMMUNOLOGY AND MOLECULAR BIOLOGY

Immune Response to a Mucosally Administered Aflatoxin B_1 Vaccine

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ABSTRACT In the present study, a mucosal vaccine was used in an effort to elicit serum IgG and intestinal secretory IgA against the mycotoxin aflatoxin B_1 (AFB) in chickens. AFB was coupled to carrier proteins (BSA and porcine thyroglobulin) for use as a vaccine and ELISA coating antigen, respectively. Seven-day-old broiler chicks were divided into groups of 10 and immunized with one of four vaccine preparations: 1) AFB-BSA conjugate alone, 2) AFB-BSA linked to the B subunit of the recombinant heat-labile enterotoxin of *Escherichia coli* (rLT-B), 3) AFB-BSA admixed with rLT-B, or 4) AFB-BSA mixed with cholera toxin (CT). Each vaccine preparation was administered perorally, intrarectally, or intraperitoneally, with a booster immunization given 2 wk later. Sera and feces were collected weekly and assayed using isotype specific ELISA. All three routes of immunization elicited significant serum IgG responses; however, the intraperitoneal route was strongest for all vaccine preparations tested. The serum IgG immune response to the AFB-BSA conjugate was enhanced by co-administration of rLT-B but not by covalent coupling to rLT-B or co-administration with CT. Secretory IgA anti-CT and anti-rLT-B antibodies were detected in fecal supernatants, but no anti-AFB responses could be detected. As all 12 treatment groups produced significant levels of serum IgG anti-AFB, any of these approaches, including oral administration without adjuvant, may afford the chicken some level of protection through simple immuno-interception of free AFB.

(*Key words*: mucosal vaccine, aflatoxin, cholera toxin, heat-labile enterotoxin, adjuvant)

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INTRODUCTION

Aflatoxin B_1 (AFB) is a mycotoxin synthesized as a secondary metabolite by the molds *Aspergillus flavus* and *Aspergillus paraciticus*. These molds establish a saprophytic infection resulting in contamination of a wide range of agricultural commodities including nuts, oilseeds and most notably corn (Trenk and Hartman, 1970; Davis et al., 1986; Payne 1998). Contamination of agricultural products by these molds can occur in pre- or postharvest crops. The primary causes of preharvest contamination are 1) growth of spores deposited on the kernel by parasitic insect vectors (Payne, 1998) and 2) airborne spores infecting corn silks that subsequently grow down the ear (Marsh and Payne, 1984). In years when drought or other stressful conditions prevail, an increased infection rate of field corn is often observed (Davis et al., 1986). Postharvest contamination occurs when storage conditions favor germination of spores that accompany grain during harvest. During storage, spores germinate and mold growth begins when the moisture content of the substrate exceeds 17.5% relative humidity and temperatures exceed 24°C (Trenk and Hartman, 1970). Thus, mycotoxin contamination of feed grains is problematic in years with a dry growing season and wet harvest season.

When aflatoxin-contaminated grain is fed to poultry, important production parameters including weight gain, feed intake, feed conversion efficiency, and reproductive performance are compromised (Hoerr, 2003). Loss in production is attributed to AFB-induced tissue damage resulting in acute or chronic illness. The pathological lesions associated with acute aflatoxicosis include hepatocellular necrosis (Bryden and Cumming, 1980), immunosuppression (Thaxton et al., 1974; Giambrone et al., 1978; Ubosi et al., 1985; Virdi et al., 1989), and alteration of renal function (Glahn, 1993), all of which are potentially lethal. Chronic exposure typically results in reduced performance, hepatic neoplasia, and increased susceptibility to

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Abbreviation Key: AAF = 2-acetylaminofluorene; AFB = aflatoxin B_1; CT = cholera toxin; DMF = dimethylformamide; GM_1 = monosialoganglioside M_1; NDV = Newcastle disease virus; PAH = polycyclic aromatic hydrocarbon; rLT-B = recombinant B subunit of the heat labile enterotoxin of *E. coli*; S-IgA = secretory immunoglobulin A; TG = porcine thyroglobulin.

a wide variety of infections (Edds et al., 1973; Brown et al., 2001). This immunosuppression is mediated at least in part by reduced phagocytic capability, decreased expression of CD 14 (the LPS receptor) on macrophages, and reduced nitric oxide production (Moon and Pyo, 2000).

Due to the ubiquitous nature of *Aspergillus sp.* in the environment, some level of mycotoxin contamination of feed grain is unavoidable. Many attempts have been made to detoxify or counteract the contamination of agricultural products including addition of aluminosilicate (Bailey et al., 1998; Ledoux et al., 1999; Miazza et al., 2000), charcoal (Edrington et al., 1997), or polyvinylpolypyrrolidone (Celik et al., 2000) binding agents to feed. In addition, other approaches such as the development of mold-resistant strains of corn (Wright et al., 2000; Brown et al., 2001), mechanical separation of contaminated grains, ammoniation, irradiation, and heating (Sinha and Bhatnagar, 1998) have been attempted. These approaches vary in the level of effectiveness, and some alter the nutrient value of the treated feed. An ideal detoxifying agent or process would inactivate the toxin, not interfere with the nutritive value of the product, and not produce or leave toxic residues behind. One alternative to treating the commodity would be to render the animal insensitive to the toxin by blocking its initial absorption by eliciting a carcinogen-specific secretory IgA (S-IgA) response (Silbart and Keren, 1989) or by simple immuno-interception using circulating antibodies (Caviezel et al., 1984).

Creech and Franks (1937) hypothesized that immunization with carcinogen-protein conjugates could inhibit tumorigenesis by eliciting a specific anti-carcinogenic humoral immune response. Subsequent work demonstrated that parenteral immunization with carcinogen-protein conjugates elicited carcinogen-specific serum antibodies in rats capable of blocking induction of tumorigenesis upon challenge (Peck and Peck, 1971). Building upon previous work, Moolten et al. (1981) suggested that a carcinogen-specific mucosal S-IgA response could block carcinogen absorption across the mucosa. Mice immunized orally and parenterally with an analogue of the polycyclic aromatic hydrocarbon (PAH) 7,12 dimethylbenzantracene covalently coupled to BSA were shown to produce intestinal immunoglobulin capable of binding the parent compound in vitro. Fecal immunoglobulin fractions isolated from immunized animals bound more radiotracer than those of control animals following oral challenge with ^{3}H-PAH. Specific binding suggests a mechanism of antibody-mediated exclusion of carcinogens. In more recent studies, rabbits receiving intestinal immunization with a 2-acetylaminofluorene (AAF) carrier protein conjugate, mixed with the mucosal adjuvant cholera toxin (CT), elicited a vigorous intestinal S-IgA anti-AAF response (Silbart et al., 1988, 1992, 1996). Additionally, passive immunization of isolated rabbit ileal segments with AAF specific S-IgA reduced transmucosal absorption of radiolabeled AAF from the lumen to the blood by 58% (Silbart and Keren, 1989).

Bacterial enterotoxins such as CT of *Vibrio cholerae* and the heat-labile toxin of *Escherichia coli* (LT) have been employed as both carrier proteins and "bystander" adjuvants for unrelated antigens delivered to mucosal surfaces (McKenzie and Halsey, 1984; Clements et al., 1988). The carrier-adjuvant properties of these molecules have been attributed to the capacity of the B subunits to bind the cell surface receptor monosialoganglioside M_1 (GM_1) and other gangliosides expressed on the surface of epithelial cells (Holmgren et al., 1974; Shida et al., 1994). Following binding of the B subunit to GM_1, the toxin's catalytic A subunit is internalized within the epithelial cell, where it disrupts intracellular signaling by catalyzing the transfer of ADP-ribose from oxidized nicotinamide adenine dinucleotide (NAD^+) to the Gsα subunit of regulatory G proteins. This results in increased levels of intracellular cAMP (Moss and Vaughan, 1977) and upregulation of the costimulatory molecule B7.2 (Cong et al., 1997). Our laboratory has reported that CT and the recombinant heat-labile enterotoxin B subunit (rLT-B) are effective carrier protein adjuvants for the carcinogen AAF (Silbart et al., 1996).

The application of rLT-B and CT as carrier proteins and adjuvants has been studied to a lesser extent in avian species, with somewhat ambiguous results. Meinersmann and Porter (1993) orally administered tetanus toxoid with and without CT to broiler chickens. Their results indicated that CT was not a mucosal adjuvant in the chicken. Similarly, CT failed to elicit a vigorous systemic or mucosal immune response when co-administered with infectious bursal disease virus (Hoshi et al., 1995). In contrast, other studies support the hypothesis that CT is a mucosal adjuvant in chickens. CT enhanced the systemic humoral immune response to a recombinant *Eimeria* antigen (Ea1A) when administered intra-intestinally. Further, conjugating Ea1A to CT elicited a stronger systemic response than co-administration of the proteins (Vervelde et al., 1998). Girard et al. (1999) noted a modest increase in duodenal and cecal IgG responses to the *Eimeria tenella* 1PE1 protein upon oral administration with CT but no overall increases in mucosal IgA. The B subunit of CT (CT-B) was shown to enhance the humoral response to inactivated Newcastle disease virus (NDV) when administered via intranasal and subcutaneous routes (Takada and Kida, 1996). Similarly, specific mucosal antibodies were generated by oral immunization of a rLT-B *Campylobacter jejuni* flagellin antigen fusion protein, resulting in a reduction in intestinal colonization upon challenge (Khoury and Meinersmann, 1995).

The current study tested the hypothesis that CT and LT-B are mucosal adjuvants in chickens by assessing the mucosal and systemic immune response to an AFB-carrier protein conjugate when administered via the mucosal route. Broiler chickens were immunized with four vaccine preparations by three different routes, and mucosal and systemic responses were subsequently measured. All 12 treatment groups produced significant levels of serum IgG anti-AFB, indicating that any of these approaches, including oral administration without adjuvant, may afford the chicken some level of protection through simple immuno-interception of free AFB.

MATERIALS AND METHODS

Birds

One-day-old male broiler chicks[3] were housed in floor pens on litter. A standard starter mash, grower mash, and water were supplied ad libitum. Chicks were allowed to acclimate for 1 wk prior to initiation of the study. All procedures, including euthanasia, were approved in advance by the University of Connecticut's IACUC committee.

Synthesis of AFB-O-carboxymethyloxime

The AFB[4] was coupled to porcine thyroglobulin (TG) following derivitization to O-carboxymethyloxime using a modification of previously published methods (Chu et al., 1977; Chu and Ueno 1977). Briefly, 100 mg of AFB was dissolved in 20 mL of pyridine-methanol-water (1:4:1) solvent at 70°C. To the AFB solution was added 182 mg of carboxymethlyamine hemihydrochloride dissolved in 10 mL of the same solvent. The reactants were continuously stirred and heated to 76°C followed by dropwise addition of the carboxymethylamine solution. The reaction was halted 90 min after the vast majority of the AFB parent compound had been converted to the oxime derivative as determined by a shift in HPLC retention time. The product was then dried under a gentle stream of nitrogen gas overnight. The dry product was dissolved in 3 mL of acetone-chloroform-acetic acid (50:45:5) and eluted over an 80cm × 2.5 cm Adsorbosil Plus silica gel column.[5] The AFB-oxime product eluted with >99% purity based upon the integrated area under the curve (compared to trace contaminants).

Conjugation of AFB-O-carboxymethyloxime to TG

The AFB-O-carboxymethyloxime (10 mg) was dissolved in 2 mL of dimethylformamide (DMF). In a separate vial, 10 mg of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide was dissolved in 2 mL of 0.05 *M* 2-(N-morpholino)ethanesulfonic acid buffer, pH 4.75, then added dropwise with stirring to the AFB-O-carboxymethyloxime DMF solution. The solution was allowed to stand at room temperature for 15 min with occasional vortexing. This solution was then added dropwise with stirring to 5 mg of TG dissolved in 6 mL of 0.05 *M* cyclohexylaminoethanesulfonic buffer, pH 9.5, in a foil-wrapped beaker. The conjugate solution was stirred for 4 h at room temperature then dialyzed against two changes of 2 L of PBS, pH 7.3, 10% DMF at 4°C followed by four changes of 2 L PBS. Removal of unconjugated AFB was determined spectrophotometrically by the disappearance of characteristic peaks at 263 and 363nm, corresponding to free AFB, in the dialysate. The protein concentration of the conjugate was determined using the Bradford method (Bradford, 1976). The molar ratio of hapten to carrier protein was estimated based on the molar absorptivity of AFB at 363 nm (20,950/M-cm^{-1}), by substitution into Beer's absorbance equation. Only conjugates with molar ratios exceeding 10 AFB moieties per 100 kDa of carrier protein were used. Each conjugate preparation was stored at –20°C until use.

Formation of AFB-O-carboxymethyloxime was determined by a shift in retention time using a Beckman ODS Ultrasphere 5 μm, 4.6 mm × 25 cm, reverse phase column.[6] Sample was eluted using a 0.05 *M* formate buffer (pH 4.0) acetonitrile gradient, 5 to 95%, over 60 min. Eluant was monitored for absorbance at 263 and 363 nm.

Conjugation of AFB-BSA to rLT-B

The AFB-BSA was coupled to rLT-B[7] by using 0.03% glutaraldehyde. AFB-BSA (5 mg) was mixed with an equal mass of rLT-B in 10 mL of PBS. Twelve microliters of 25% glutaraldehyde was added to the solution and allowed to stir for 2 h at 37°C. Glycine (125 μL of a 1 *M* solution) was added to inactivate unreacted aldehydes, and the solution was then stirred for an additional 30 min followed by dialysis overnight against 2 L of PBS at 4°C. The precipitate was removed by centrifugation at 1,000 × *g* for 15 min. The protein concentration of the supernatant was then determined by the Bradford method (Bradford, 1976).

Conjugation of AFB-BSA to rLT-B was determined by HPLC molecular sieve chromatography using a Bio-Sil SEC-250 gel filtration column.[8] Samples were applied to the column in 0.05 *M* phosphate buffer, pH 7.3, prior to and following conjugation. Eluant was monitored for absorbance at 280 nm.

Experimental Design

Chickens were divided into 12 treatment groups containing 10 birds per group. Each of four vaccine preparations AFB-BSA, AFB-BSA mixed with rLT-B (AFB-BSA+rLT-B), AFB-BSA coupled to rLT-B (AFB-BSA-rLT-B), and AFB-BSA mixed with CT (AFB-BSA+CT) was administered via one of three routes: oral, intrarectal, or intraperitoneal. The mass of AFB-BSA was maintained at 100 μg per dose in each vaccine preparation. Additionally, the mass of rLT-B, either covalently coupled or admixed, was also administered at 100 μg per dose. For the AFB-BSA+CT preparation, a dose of 100 μg AFB-BSA was mixed with 10 μg of CT and administered via the three routes described above without any overt signs of toxicity. Oral vaccines were administered in a total volume of 0.5 mL oral immunization buffer (7.5% wt/vol sodium carbonate buffer, pH 9.0). Chickens were orally immu-

[3]Arbor Acres Farms, Glastonbury, CT.
[4]All reagents were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, unless otherwise noted.
[5]Alltech Associates, Inc., Deerfield, IL.
[6]Beckman Insturments Inc., San Ramon, CA.
[7]Kindly supplied by John Clements, Tulane University.
[8]Bio-Rad Laboratories, Richmond, CA.

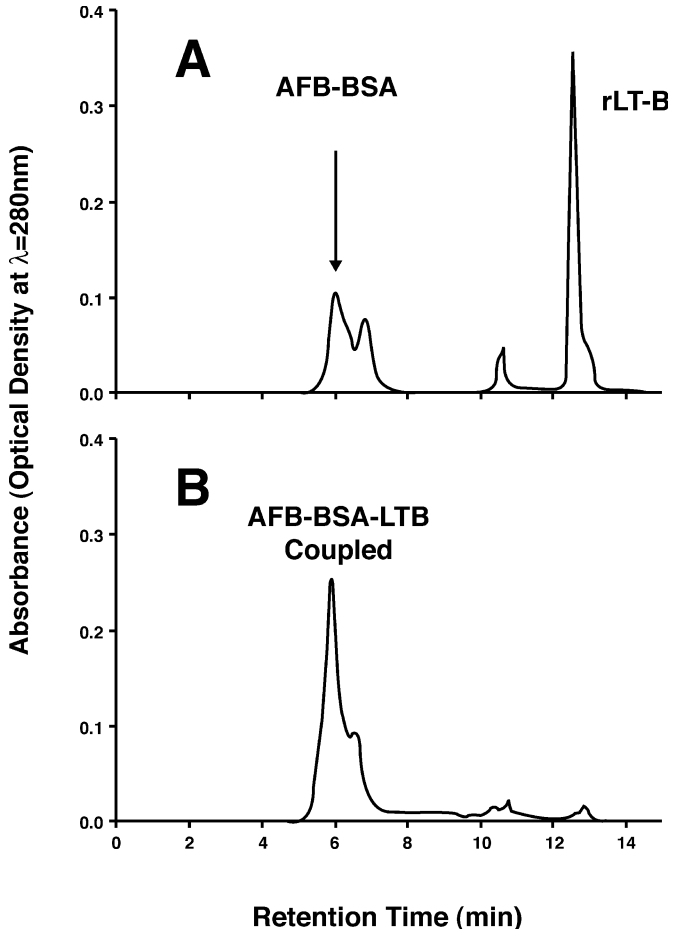

FIGURE 1. HPLC elution profile of aflatoxin B_1 (AFB) conjugates separated by molecular sieve chromatography. AFB conjugates were injected onto a Bio-Sil SEC-250 gel filtration column (Bio-Rad Laboratories, Richmond, CA), and the eluant was monitored at λ = 280 nm. Unconjugated AFB-BSA mixed with 100 μg of recombinant B subunit of the heat-labile enterotoxin of *Escherichia coli* (rLT-B; panel A) revealed two distinct peaks corresponding to AFB-BSA and rLT-B. The glutaraldehyde coupled material (AFB-BSA-LTB) eluted as a single peak (panel B) with only a trace amount of rLT-B remaining uncoupled.

nized by pipetting the vaccine into the oral cavity, intraperitoneally by injection, and intrarectally by inserting a 22-ga blunt-tipped feeding needle through the cloaca into the rectum. The primary immunization was administered at 7 d of age, followed by a booster immunization 2 wk later. Samples of sera and fecal pellets were collected from each animal beginning at 7 d of age and then weekly thereafter for the duration of the study. A cohort preimmunization sample of sera and fecal pellets was collected from 10 hatchmates and pooled.

ELISA

The ELISA were performed as previously described (Silbart et al., 1988) with minor modifications. Dynatech Immulon 4 microtiterplates[9] were coated with AFB TG conjugate (AFB-TG), CT, rLT-B, and TG control protein, 10 μg/mL, in coating buffer (0.05 *M* bicarbonate buffer, pH 9.8) and then tightly covered with two layers of Parafilm. Coated plates were incubated overnight at 25°C then stored at 4°C until use. Sera were diluted 1:50 in PTA (50 m*M* $KHPO_4$, 0.1% Tween-20, 0.02% sodium azide, pH 7.4) for anti-AFB antibody assays. Fecal samples were suspended in 1:5 wt/vol PBS, vortexed vigorously for 3 min, and then centrifuged at 200 × *g* for 15 min at 4°C. The supernatant was diluted 1:2 in PTA and assayed for CT, rLT-B, or AFB specific S-IgA. The diluted samples were applied to microtiter plates at 100 μL per well, incubated at 25°C for 2 h, and then washed five times with PTA using an EL 403 Bio-Tek microplate washer.[10] The appropriate alkaline phosphatase conjugated antibody was applied (goat anti-chicken IgG[4] or goat anti-chicken IgA[11]) and incubated for 2 h at 25°C. After being washed with PTA, the plates were developed using 1 mg/mL p-nitro phenyl phosphate (pNPP) substrate in 0.05*M* carbonate buffer, 1 m*M* $MgCl_2$, pH 9.8. Product formation was measured at 15, 30, and 60 min using a Bio-tek EL-311 microplate reader[10] (λ = 405 nm). A positive control was included on each plate so that interplate variation could be normalized by correcting the values on each

[9]Dynex Technology, Chantily, VA.
[10]Bio-Tek Instruments, Inc., Winooski, VT.
[11]Bethyl Laboratories, Montgomery, TX.

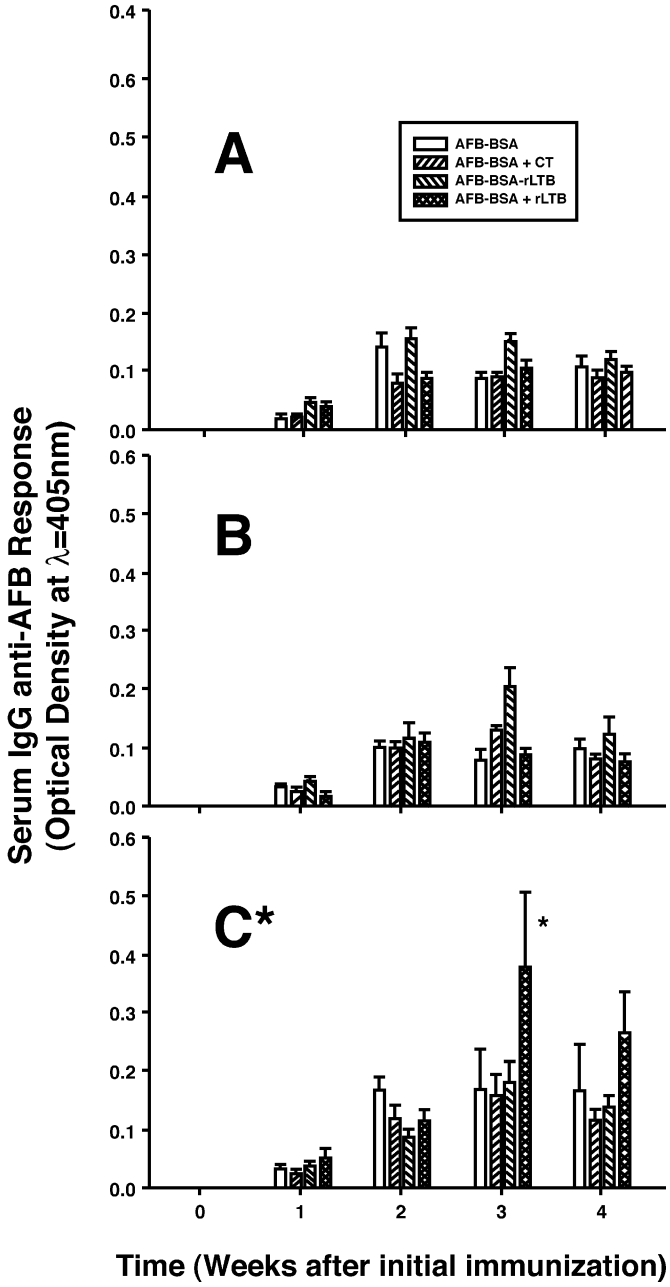

FIGURE 2. Serum IgG anti-aflatoxin B_1 (AFB) responses following immunization by three different routes. Groups of chickens were immunized using one of four conjugate preparations (see legends) and their sera analyzed by isotype specific ELISA. Birds immunized via the oral route (panel A) and intrarectally (panel B) were roughly comparable in the AFB-specific responses, whereas those immunized intraperitoneally (panel C) were significantly (*$P < 0.05$) higher. Within the intraperitoneally immunized group, birds receiving the AFB-BSA conjugate plus recombinant B subunit of the heat-labile enterotoxin of *Escherichia coli* (rLT-B) demonstrated a significantly increased response (*$P < 0.05$) as well. CT = cholera toxin.

plate to the aggregate mean of the positive control on all plates. The positive control antiserum was obtained from a chicken immunized intramuscularly with AFB-BSA emulsified in complete Freund's adjuvant. The negative control consisted of the pre-immune cohort samples. Specific response was determined by subtracting the mean of the TG control wells from the mean of the corresponding AFB-TG wells for each test sample. These net values were normalized to the overall group mean derived from the positive control on each plate.

To confirm AFB-specific responses, competitive ELISA were performed using AFB-TG, TG-coated microtiter plates in a similar manner as above. Antisera were incubated with free AFB at 10, 1, 0.1, 0.01, or 0 μg/mL for 15 min prior to application on the microtiter plate. Percentage inhibition was determined by dividing the values from wells containing the free AFB by those that contain no free AFB.

To confirm GM_1 binding ability and AFB haptenation of the AFB-BSA-rLT-B vaccine preparation, a GM_1 ELISA was performed using microtiter plates coated with 10 μg/mL GM_1 and TG. The AFB-BSA-rLT-B conjugate was applied at 100 μg/mL and incubated for 2 h at room temperature. Plates were washed with PTA followed by application of anti-AFB control antibody as well as free AFB in the competitive ELISA format. Bound anti-AFB antibody was determined using alkaline phosphatase-labeled goat anti-chicken IgG.

Statistical Analysis

Statistical analysis of differences between treatments was performed using PROC MIXED function of SAS software.[12] Treatments were organized in a three by four factorial completely random design. An unstructured covariance matrix yielded the highest Akaike's information criteria values and Schwartz's Bayesian criteria. These criteria determine fit for the covariance matrix. Comparisons between treatments were made using least squares means. Differences were considered statistically significant at an $\alpha < 0.05$.

RESULTS

Characterization of Coupling AFB-BSA to rLT-B:

Conjugate that bound GM_1 and AFB antibodies in the GM_1 ELISA was further analyzed by HPLC molecular sieve chromatography to determine the extent of glutaraldehyde-mediated coupling of AFB-BSA to rLT-B. Figure 1 shows the retention times of AFB-BSA mixed with rLT-B (panel A) and AFB-BSA covalently coupled to rLT-B (panel B). The mass of AFB-BSA and rLT-B was kept equal in both injections. AFB-BSA eluted at 6 min and rLT-B eluted at 12.5 min. The coupled product eluted at 5.9 min. The decrease in retention time and increase in peak area indicate an increase in molecular weight for the coupled product as does the disappearance of the rLT-B peak. The coupled product bound specifically to plates precoated with GM_1. In addition competitive ELISA experiments indicated that anti-AFB antibody

[12]Release 6.09, SAS Institute Inc., Cary, NC.

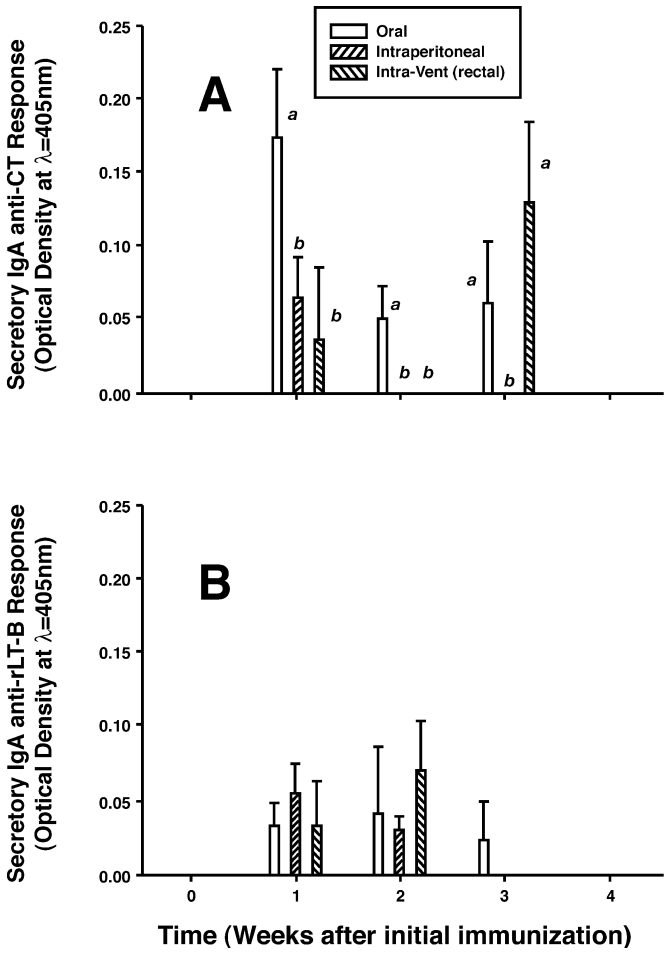

FIGURE 3. Secretory IgA responses in feces. Fecal samples were collected and solubilized in PBS and then assayed by isotype-specific ELISA. Birds receiving cholera toxin (CT; panel A) or recombinant B subunit of the heat-labile enterotoxin of *Escherichia coli* (rLT-B; panel B) as adjuvants to the AFB-BSA conjugates produced detectable amounts of S-IgA anti-CT or rLT-B, respectively, but neither adjuvant augmented the immune response with regard to the AFB hapten group (see text). Groups in panel A with different letters were statistically different ($P < 0.05$). No statistical differences were noted among groups in panel B.

binding was inhibited by as much as 94% when preincubated with free AFB (data not shown).

Serum Anti-AFB IgG Response

The serum IgG anti-AFB responses did not differ between groups of chickens vaccinated by the oral or intrarectal route (Figure 2A and B, $P > 0.05$). Conversely, immunizing via the intraperitoneal route yielded a greater ($P < 0.05$) IgG response than either mucosal route. The strongest response was elicited by AFB-BSA+rLT-B when administered by the intraperitoneal route and was greater ($P < 0.05$) than all other treatment groups. Sera from the three highest responding chickens within the AFB-BSA+rLT-B intraperitoneally immunized group were subjected to competitive ELISA and indicated that >80% of the binding was AFB-specific (data not shown). Sera IgG from these three birds competed at a level comparable to that of control sera. Also, the peak response occurred at wk 3, indicating that boosting augmented the immune response in the AFB-BSA+rLT-B intraperitoneal group. Serum IgG titers were determined for the highest responding chicken from each treatment group. In keeping with the results shown in Figure 2, the highest titers were observed when vaccine preparations were administered via the intraperitoneal route (data not shown).

Intestinal Secretory IgA Response to CT and rLTB

No AFB specific S-IgA was detected in the fecal supernatants from any of the treatment groups. However, anti-CT and anti-rLT-B S-IgA were detected by indirect ELISA in fecal supernatants (Figure 3). There was no difference between responses to CT and rLT-B using the routes tested. Within the groups that received AFB-BSA+CT, the orally and intrarectally immunized birds demonstrated greater response ($P < 0.05$) to CT than did the intraperitoneal group. There was no difference among routes of administration within any of the groups receiving rLT-B. In addition, responses were not augmented by booster immunization, nor did S-IgA response increase over time, in keeping with previous studies (Silbart et al., 1996).

DISCUSSION

Immunization via the intraperitoneal route elicited a significantly stronger anti-AFB serum IgG response ($P < 0.05$), in comparison to the oral and intrarectal routes for each of the vaccine preparations tested. In addition, the AFB-BSA+rLT-B vaccine preparation was superior to all other treatments at eliciting an anti-AFB serum IgG response. The current study indicates that the intraperitoneal route was superior to the other routes tested, in keeping with the results of Muir et al., (1995), who showed that priming via the intraperitoneal route followed by oral boosting generates a strong S-IgA response against nonreplicating antigens.

The results of this study indicate that CT does not act as a mucosal adjuvant for AFB conjugates in chickens. However, CT and rLT-B did elicit an intestinal S-IgA response when administered by the oral and intrarectal routes. The mucosal unresponsiveness to AFB agrees with previous work in which CT failed to enhance the mucosal immune response to orally administered tetanus toxiod (Meinersmann and Porter, 1993). Our work also supports the finding that CT failed to enhance the serum IgG response to orally administered antigens. Taken together, it appears that CT is a much more potent mucosal adjuvant in mammals than in chickens. The reason for lack of adjuvant activity by CT in chickens is currently unknown and may be related to unique immunostimulatory pathways or the local microenvironment within mucosal inductive sites. Curiously, chickens have been shown to express receptors that bind CT on the surface of intestinal epithelial cells, and intracecal administration of CT has resulted in infiltration of the lamina propria by TCR2+, CD4+, and CD8+ T lymphocytes (Vervelde et al., 1998). Chickens receiving intracecal immunization with Ea1A and CT demonstrated a serum anti-Ea1A and anti-CT IgG response. Unfortunately, mucosal S-IgA responses were not assessed in that study.

Other studies have indicated that the B subunits of bacterial enterotoxins are better adjuvants than holotoxins in chickens. Khoury and Mienersmann (1995) found that oral administration of a rLT-B *Campylobacter jejuni* flagellin fusion protein inhibited colonization upon challenge. Herein, we showed that coupling to rLT-B did not enhance anti-AFB responses; however, simple admixing was effective. In addition, intranasal immunization of inactivated NDV with CT B subunit (CT-B) protected chickens from challenge with a lethal strain of NDV (Takada and Kida, 1996). Commercially available CT-B was used in the NDV study, which might have contained trace amounts of the A subunit. Thus, it appears that the B subunit is capable of enhancing serum responses but that optimum response may require trace amounts of the catalytic A subunit. These results indicate that further work on induction of the avian mucosal immune system is warranted and may yield better mucosal adjuvants than are currently available.

In the present study, we were able to induce an AFB specific serum response using all of the vaccine preparations and routes of administration. The mucosal route of immunization, especially oral and nasal, is a very desirable route of vaccine administration due to its ease and lack of invasiveness. These results emphasize the potential of immunizing chickens with nonreplicating antigens via the mucosal route. The humoral responses observed in this study could most certainly be optimized and may very well confer protective immunity.

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