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Mucosal unresponsiveness to aflatoxin B_1 is not broken by cholera toxin

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Summary Rabbits immunized via chronically isolated ileal loops with aflatoxin B_1 (AFB) conjugated to porcine thyroglobulin (TG) mixed with the mucosal adjuvant cholera toxin (CT) produced very small mucosal antibody responses to AFB. Strong mucosal and systemic antibody responses to CT and TG were generated by this immunization protocol, suggesting that the observed unresponsiveness was specific to AFB. Parenteral immunization with AFB–TG produced strong serum IgG anti-AFB responses, indicating that the conjugate preparation was immunogenic and that the rabbits possess the requisite systemic B and T cell repertoires to recognize and respond to AFB. This mucosal unresponsiveness was distinct from oral tolerance, as animals immunized mucosally with AFB–TG mixed with CT produced vigorous serum IgG anti-AFB responses upon subsequent parenteral immunization with AFB–TG. *In vitro* mitogen stimulation of lymphocytes isolated from Peyer's patches and mesenteric lymph nodes of unimmunized rabbits revealed the presence of AFB-specific B cells at levels comparable with those found in the spleen. These observations indicate that unresponsiveness to AFB is hapten-specific, restricted to the mucosa, and refractory to the adjuvancy of CT.

Key words: aflatoxin B_1, cholera toxin, mucosa, secretory IgA, tolerance.

Introduction

Moolten *et al.* performed the first studies aimed at eliciting mucosal antibodies (Ab) to block carcinogen absorption.[1,2] Mice immunized parenterally and orally with an analogue of the polycyclic aromatic hydrocarbon (PAH) 7,12-dimethylbenzanthracene covalently coupled to BSA generated intestinal immunoglobulin capable of binding the parent compound *in vitro*. Additionally, the faecal Ig fraction from immunized animals bound more radiolabel than that of control animals following oral challenge with [^{3}H]-PAH,[1] suggesting a mechanism of Ab-mediated carcinogen clearance. In more recent studies, mucosal immunization of rabbits via chronically isolated ileal loops with a 2-acetylaminofluorene (AAF) derivative conjugated to carrier proteins and mixed with the mucosal adjuvant cholera toxin (CT) generated a vigorous intestinal secretory IgA (S-IgA) anti-AAF response.[3–5]

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Furthermore, passive immunization of isolated ileal segments with S-IgA anti-AAF reduced transmucosal absorption of radiolabelled AAF from the lumen by 58%.[4]

Cholera toxin is an enterotoxin produced by *Vibrio cholerae* which, unlike most protein Ag, is a potent mucosal immunogen.[6,7] Cholera toxin is also a mucosal adjuvant when administered orally with proteins, viruses, and hapten-carrier protein conjugates.[5–9] It exerts a number of immunomodulatory effects, including: (i) upregulation of Ag presentation and IL-1 secretion by macrophages;[10,11] (ii) promotion of B cell isotype switching from IgM to IgG and IgA;[12,13] and (iii) increased priming of T cells and induction of a Th2 type cytokine profile, concomitant with inhibition of Th1 type responses in the gut.[14–16]

Although CT can block the induction of mucosal and systemic tolerance to co-administered Ag,[6,7] its ability to abrogate an established mucosal or systemic tolerance remains unresolved. Oral priming with keyhole limpet haemocyanin (KLH) followed by an oral boost with a mixture of KLH and CT resulted in a markedly diminished mucosal Ab response to KLH relative to that seen when both priming and boosting doses contained CT.[8] Similar results were obtained using ovalbumin (OVA) along with the heat-labile enterotoxin of *E. coli* (LT).[17] These data suggest that bacterial enterotoxins may not be effective mucosal adjuvants for Ag to which tolerance has already been established.

Aflatoxin B_1 (AFB), a secondary metabolite produced

by several moulds, most notably *Aspergillus flavus*, is an ubiquitous contaminant of many food crops. In preliminary studies we observed an unexpectedly small intestinal S-IgA anti-AFB response following mucosal immunization of rabbits via isolated ileal loops with AFB–carrier protein conjugates mixed with CT. These results contrast with those of our previous studies in which similar conjugates prepared using AAF as the hapten generated strong intestinal S-IgA responses to the AAF determinant when used in a similar immunization protocol.[3–5,18]

The goals of the present study were to determine if mucosal unresponsiveness to AFB: (i) could be broken by CT; (ii) was restricted to the mucosal arm of the immune system; and (iii) was specific to the AFB determinant or indicative of a more generalized mucosal immunosuppression.

Materials and methods

Animals, surgery and immunization

Specific pathogen-free female New Zealand White rabbits (2.6–3.0 kg, Hazleton Research Products, Denver, PA, USA) were housed for at least 1 week prior to surgery; fed 125 g/day of Purina Certified High Fiber Rabbit Diet HF5326 (Purina Mills, St Louis, MO, USA) and provided with water *ad libitum*. All animal use protocols were approved by the University of Connecticut's Institutional Animal Care and Use Committee. Chronically isolated ileal loop surgery (Thiry-Vella) was performed essentially as described by Keren *et al.*[19] with minor modifications. Animals were fasted for 24 h prior to surgery and anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). A midline incision was made to expose the terminal ileum, and a 15 cm section containing one Peyer's patch (PP) was excised, leaving the surrounding vasculature intact. The mesentery was then incised to allow movement of the excised segment and intestinal continuity was restored by end-to-end anastomosis. Each end of the excised segment was surgically attached to a length of plastic silastic tubing, which was anchored to the body wall near the site of the midline incision. The tubing was tunnelled subcutaneously and exteriorized near the scapulae. The incision was then closed in two layers and the animals were fitted with plastic collars to prevent damage to the tubes. Ileal secretions were collected daily by flushing the loop with 10 cm^3 of air from a syringe and collecting the fluid expelled from the opposite tube. The loop was then flushed with 10 cm^3 of sterile saline, and finally with 10 cm^3 of air, leaving it empty.[20] Collected secretions were centrifuged for 5 min at $200 \times g$ at room temperature to remove mucus, and supernatants were collected and stored at −20°C until assay.

Mucosal immunizations were administered following flushing of the ileal loops, generally within 2 days of surgery. Priming doses consisted of 100 μg of AFB–TG or unconjugated TG, with or without 100 μg of CT (Azide-free holotoxin, List Biological Laboratories, Campbell, CA, USA), in a total volume of 4 mL of PBS, followed by three identical weekly boosts. This dose of CT (expressed as a milligram of CT per kg bodyweight basis) is about one-tenth of the dose customarily administered to mice. Intramuscular priming immunizations consisted of 100 μg of AFB–TG or unconjugated TG in PBS emulsified with an equal volume of CFA. A total volume of 1 mL was injected into four locations over the hips followed by a similar dose in IFA 4 weeks later. Animals were bled via the auricular artery prior to immunization and weekly thereafter. Blood was stored overnight at 4°C to allow clotting, centrifuged ($400 \times g$, 10 min, room temperature), and the serum fraction aspirated and stored at −20°C until assay. The immunization schedule is shown in Table 1.

Synthesis of AFB-O-carboxymethyloxime

In order to couple AFB to carrier proteins, the parent molecule was first derivatized to the *O*-carboxymethyl oxime using a modification of the methods of Chu *et al.*[21,22] and Langone *et al.*[23] Briefly, 92 mg of AFB (0.29 mmol, Sigma Chemical Co., St Louis, MO, USA) was dissolved in 25 mL of a pyridine–methanol–water (1 : 4 : 1) solvent system. To this solution 121.8 mg of carboxymethoxylamine hemihydrochloride was added (0.55 mmol, Aldrich Chemical Co., Milwaukee, WI, USA), and the mixture was heated to 80°C for 3 h with stirring in a round bottom flask protected from light. The reaction was monitored by thin layer chromatography (TLC) using a glacial acetic acid–acetone–chloroform solvent system (5 : 45 : 50) in which AFB travelled with an R_f value of 0.65, while the oxime travelled at an R_f of 0.53. After cooling, the solvent was removed using a gentle stream of dry nitrogen gas overnight. The solid was then taken-up in the TLC solvent system and applied to an Adsorbosil Plus silica gel column previously equilibrated with the same solvent system (10 m, 0.8×60 cm, Alltech Associates, Deerfield, IL, USA). The two main fractions which eluted immediately after the unreacted AFB were combined and the solvent was removed by rotoevaporation. The solid was taken-up in

Table 1 Immunization schedule

Group	Immunization	*N*	Route	Booster immunizations
I	AFB–TG + CT	5	IL	Days 7, 14, 21
II	TG + CT	5	IL	Days 7, 14, 21
III	AFB–TG	5	IL	Days 7, 14, 21
IV	TG	2	IL	Days 7, 14, 21
V	AFB–TG + CT/AFB–TG	3	IL/i.m.	Days 7, 14, 21; 25, 55
VI	AFB–TG	3	i.m.	Day 30
VII	TG	2	i.m.	Day 30

Intra-loop (IL) immunizations consisted of a priming dose of 100 μg each of conjugate or Thyroglobulin (TG) with 100 μg of cholera toxin (CT), as indicated, via chronically isolated ileal loops, in PBS, followed by three identical booster doses. Intramuscular immunizations consisted of a priming dose of 100 μg of aflatoxin B_1–TG or TG emulsified in CFA and a booster dose 1 month later in IFA. All animals were given a priming dose on day 0. Group V animals were immunized IL with AFB–TG mixed with CT, and subsequently twice i.m. with AFB–TG in CFA/IFA as described in materials and methods.

water (pH 10), then twice extracted with an equal volume of ethyl acetate. The aqueous layer was then acidified to pH 2 with concentrated HCl, and the precipitate was vacuum filtered to give about 57% yield of pure AFB–oxime. The identity of the purified product was confirmed by [^{3}H]-NMR, infrared spectroscopy, mass spectroscopy and HPLC, with results in agreement with Chu *et al.*[22]

AFB conjugates

To produce the conjugates, 20 mg of AFB-*O*-carboxymethyl oxime was dissolved in 2 mL of dimethylformamide (DMF). Twenty milligrams of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) dissolved in 2 mL of 0.5 mol/L 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 4.75, was added to the AFB–*O*-carboxymethyl oxime dropwise with stirring. The solution was allowed to stand at room temperature for 15 min with occasional vortexing. This solution was then added dropwise with stirring to a solution of 10 mg of TG in 6 mL of 0.5 mol/L cyclohexylaminoethane sulfonic acid (CHES) buffer, pH 9.5, in a foil wrapped beaker. This solution was covered and stirred gently for 4 h at room temperature. The conjugate solution was then dialyzed against two changes of 2 L of 50 mmol/L PBS (pH 7.3, 10% DMF) at 4°C and four changes of 2 L of PBS. Removal of unconjugated AFB from the conjugate preparation was determined spectrophotometrically by the disappearance of the characteristic peaks at 263 and 363 nm in the dialysate. The protein concentration of the resulting conjugate solution was determined by Bradford assay[24] using a BSA standard curve. The concentration of conjugated AFB was estimated based on the molar absorptivity of unconjugated AFB at 363 nm (20 950), by substitution into Beer's absorbance equation. Only conjugates with molar ratios exceeding 10 AFB moieties per 100 kDa of carrier protein were used. The conjugate preparation was then aliquotted and stored at – 20°C until use.

ELISA

Microtitre plates (Immulon 4, Dynatech, Chantilly, VA, USA) were coated with AFB–BSA conjugate, TG, BSA (Sigma, St Louis, MO, USA), or CT, at 10 μg/mL in carbonate buffer (0.05 mol/L, pH 9.6). Plates were incubated overnight at room temperature and stored at 4°C until use.

Serum or intestinal secretions diluted in wash buffer (0.05 mol/L Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 and 0.1% BSA) was added to plates and incubated at room temperature for 1 h. Plates were washed eight times with wash buffer using a microplate washer (Biotek EL 311, Winooski, VT, USA) followed by the addition of affinity purified isotype specific secondary Ab (biotinylated goat anti-rabbit IgA or alkaline phosphatase-labelled goat anti-rabbit IgG) diluted in wash buffer and incubated overnight at 4°C. Following another wash, streptavidin–alkaline phosphatase diluted in wash buffer was added to plates containing biotinylated secondary Ab after washing. Plates were developed using the Immunoselect ELISA Amplification System (Gibco BRL, Gaithersburg, MD, USA) to maximize the sensitivity of the assay. The substrate was incubated at room temperature for 20 min prior to addition of amplifier solution, and the absorbance was measured at 490 nm 5, 10 and 20 min after addition of the amplifier solution. All assays were performed in duplicate and positive and negative controls were included on all plates. The positive control consisted of pooled serum or intestinal secretions as appropriate from responding animals used at a dilution that yielded an approximate net OD of 1.0; the negative control consisted of pooled serum or intestinal secretions from unimmunized animals assayed at the same dilution as the serum or secretions in question. The net OD was calculated as the average 20 min OD of duplicate wells containing the relevant Ag minus the average OD of duplicate BSA-coated wells. To compensate for between-plate variability, individual plates were normalized to the mean of the appropriate positive control. Titre was operationally defined as the highest $\log_2$ dilution giving a net OD greater than twice the background OD. The Ag specificity of ELISA responses was verified by competition with excess Ag in the liquid phase (data not shown).

Preparation of cell suspensions

Unimmunized animals were killed by sodium pentobarbital overdose and a midline abdominal incision was made to expose the small bowel. Spleens (SP) and mesenteric lymph nodes (MLN) were located, aseptically removed, and placed in individual dishes of incomplete medium (Iscoves Modified Dulbecco's Medium containing 2 mmol/L-glutamine, 50 μmol/L of 2-ME and 50 μg/mL of gentamycin). Peyer's patches were removed, trimmed with small scissors, and placed in incomplete medium. After removal of PP, about 20 cm of small intestine was placed in Ca^{2+} and Mg^{2+}-free HBSS containing antibiotic–antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin, 0.025 μg/mL amphotericin B, Gibco BRL). Spleen, PP and MLN were transferred to sterile incomplete medium. Splenic lymphocytes were obtained by injecting incomplete medium into the organ with a 22 gauge needle. Mesenteric lymph nodes and PP were minced with small scissors, pressed between frosted glass slides to dissociate cell clumps, filtered through a fine mesh screen and allowed to settle for 5 min to remove debris. Cells were washed twice by centrifugation ($200 \times g$, 10 min, room temperature) and resuspended in incomplete medium. Cells suspended in complete medium (incomplete medium with antibiotic–antimycotic and 10% FCS (Gibco BRL, Gaithersburg, MD, USA) added) were underlaid with 40 and 60% Percoll and centrifuged at $1900 \times g$ for 13 min. Lymphocytes were collected from the 40:60 interface, washed three times as above, and resuspended in complete medium. Cells were then counted with a haemacytometer. Cell viability was consistently greater than 95% by trypan blue dye exclusion.

Lamina propria (LP) lymphocytes were obtained by a modification of the method of Davies and Parrott.[25] Following removal of PP, about 20 cm of the small intestine was flushed with incomplete medium and transferred to a dish containing HBSS with antibiotic–antimycotic and 5 mmol/L EDTA. Tissue was sliced longitudinally, cut into 0.5 cm segments and gently stirred at room temperature. Medium was drawn off and replaced as it clouded with shed epithelial cells. Tissue segments were then incubated with shaking in HBSS containing 20 U/mL of collagenase (CLS 2, Worthington Biochemical, Freehold, NJ, USA) and 5% FCS for 90 min at room temperature. The supernatant was then drawn off and centrifuged ($200 \times g$, 10 min, room temperature), and the cell pellet was resuspended in incomplete medium. Cells were washed, fractionated by Percoll gradient separation, and assayed for viability as described above.

Mitogen stimulation

Lymphocytes (2×10^6 cells/mL) in complete medium were cultured with 50 μg/mL of LPS from *S. enteritidis* (3126-25, Difco, Detroit, MI, USA) and 2.5 μg/mL of PWM (Sigma) for 4 days (37.5°C, 5% CO_2 in humidified air). This protocol was found to

induce maximal proliferation measured by [^{3}H]-thymidine incorporation and cell counting (data not shown). Cells were fed on days 2 and 4 by removing half of the medium and replacing it with an equal volume of complete medium. Control cells were cultured under identical conditions without mitogen. On day 4, cells were harvested for ELISPOT analysis.

Enzyme-linked immunosorbent spot assay (ELISPOT)

The ELISPOT technique was adapted from Czerkinski[26] with modifications. Sterile 96-well nitrocellulose bottom plates (MAHA S45, Millipore, Bedford, MA, USA) were wetted with PBS, coated with 50 μL per well of AFB-BSA, BSA, or TG diluted to 20 μg/mL in PBS, and incubated overnight at room temperature. Plates were then washed three times with PBS, blotted dry with paper towels, and blocked with complete medium for 1 h at room temperature. Complete medium (100 μL) containing 2×10^6 viable cells/mL was added to each well and the plates were incubated overnight (37.5°C, 5% CO_2). LP lymphocytes were cultured at 1×10^6 viable cells/mL. Plates were then washed three times with phosphate buffer (0.05 mol/L, pH 7.4) containing 0.1% Tween 20 and 0.02% sodium azide (PTA). Goat anti-rabbit Ig (50 μL per well, biotinylated in our laboratory[27]), diluted in PTA, was added and the plates were incubated overnight at room temperature. Plates were washed three times with PTA, and 50 μL of streptavidin–horseradish peroxidase (Amersham, Arlington Heights, IL, USA) diluted in PTA was added to each well and incubated for 1 h at room temperature. Plates were washed three times with PTA and developed with 200 μL per well of the chromogen 3-amino-9-ethylcarbazole (AEC, Sigma A-6926) prepared according to the manufacturer and filtered through a 0.45 micron filter prior to use. After incubating for several hours, the number of antibody secreting cells (ASC) was scored using a dissecting microscope at 400 × magnification. Enumeration of anti-AFB ASC was performed by subtracting the mean number of ASC in duplicate BSA-coated wells from the mean number of ASC in duplicate AFB–BSA-coated wells. Anti-TG ASC were calculated by subtracting the mean ASC in duplicate uncoated wells from the ASC in duplicate TG-coated wells. ASC were converted to ASC per 10^6 expanded cells. Data are expressed as the mean ± SEM of ASC from groups of three or four animals.

Statistics

Data are expressed as the arithmetic mean ± SEM. The SAS (SAS Institute, Cary, NC, USA) General Linear Models procedure was used to compute analyses of variance. The LSMEANS option within this procedure was used to analyse peak day differences between the treatment groups. Student's *t*-test was used to determine differences between means of two groups. In all cases, a P value of <0.05 was used as the threshold of significance.

Results

Mucosal unresponsiveness to AFB is not broken by CT

When AFB–TG conjugates mixed with CT were administered to rabbits via isolated ileal loops, a minimal S-IgA anti-AFB response was detected in ileal secretions assayed at 4-day intervals (Fig. 1). This response was barely detectable by ELISA using *p*-nitrophenyl phosphate as the substrate, and required the use of an amplified ELISA protocol in order to quantify the response. Although statistically significant differences were noted at days 4 and 40, this response was not significantly different overall from that of animals immunized with AFB–TG alone ($P = 0.12$; Fig. 1), demonstrating that CT did not act as an adjuvant for AFB. The peak intestinal S-IgA anti-AFB titre for this group was 8 (2^3, Table 2). Mucosal immunization with AFB–BSA conjugates mixed with CT produced similar results (data not shown).

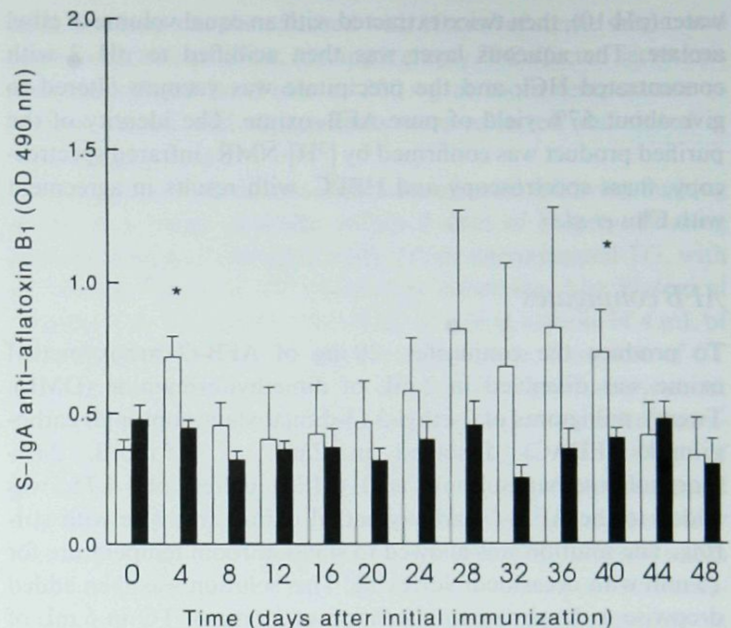

Figure 1 Mucosal S-IgA anti-aflatoxin B_1 (AFB) response. Animals ($n = 5$) were primed with (■) 100 μg of aflatoxin B_1–thyroglobulin (AFB–TG) and (□) 100 μg of AFB–TG + 100 μg of cholera toxin, via chronically isolated ileal loops on day 0 and given identical booster immunizations on days 7, 14 and 21. The data points indicate the mean response (SEM). Intestinal secretions at 4 day intervals were assayed by amplified ELISA at a dilution of 1 : 8. Asterisks denote a significant difference between the groups ($P < 0.05$).

Mucosal unresponsiveness to AFB is hapten-specific

In order to differentiate between hapten-specific unresponsiveness and generalized immunosuppression, a group of animals was mucosally immunized with unconjugated TG mixed with CT (Table I, Group II). The intestinal S-IgA anti-CT and anti-TG responses of these animals were comparable with those of animals immunized with AFB–TG plus CT (Fig. 2), ruling out AFB-mediated immunosuppression. Also, there were no significant differences in the S-IgA anti-TG responses attributable to conjugation with AFB ($P = 0.33$). The intestinal S-IgA anti-TG response of animals immunized with AFB–TG plus CT, or TG plus CT, was significantly greater than that of animals immunized with AFB–TG or TG alone ($P = 0.0003$), demonstrating the adjuvancy of CT for the anti-TG response (Fig. 2).

Animals immunized via ileal loop with AFB–TG mixed with CT produced a small serum IgG anti-AFB response, with an average titre of 16 (2^4, Table 2). This protocol did not generate measurable intestinal IgG or

Table 2 Log base 2 peak titres (mean ± SEM) following mucosal and/or systemic immunization with aflatoxin B_1-carrier protein conjugates

Group	Serum IgG	Serum IgA	Intestinal IgG	Intestinal IgA
Anti-TG titre				
I	15 ± 0.7	5 ± 0.6	7 ± 0.6	13 ± 0.4
II	16 ± 0.2	5 ± 0.5	8 ± 0.9	13 ± 0.6
III	<3	<3	<3	3 ± 0.1
IV	<3	<3	<3	4
V	19 ± 0.3	<3	ND	ND
VI	19 ± 0.3	<3	NA	NA
VII	22 ± 0.7	<3	NA	NA
Anti-CT titre				
I	18 ± 1	5 ± 0.7	10 ± 0.7	14 ± 0.4
II	20 ± 0.5	5 ± 0.5	11 ± 0.6	14 ± 0.5
Anti-AFB titre				
I	4 ± 0.4	<3	<3	3 ± 0.2
III	<3	<3	<3	<3
V	15 ± 0.7	<3	ND	ND
VI	15 ± 1.3	<3	NA	NA

Group numbers are as shown in Table 1. The intestinal and serum IgA and IgG anti-thyroglobulin and anti-cholera toxin responses of group I were not significantly different from those of group II as measured by two-tailed Student's t-test ($\alpha = 0.05$). The serum IgG anti-thyroglobulin responses of groups V and VI were not significantly different from each other or from that of group VII by t-test. ND, experiment not done. NA, not applicable (chronically isolated ileal loop surgery was not performed on these animals).

serum IgA anti-AFB responses (Table 2). This immunization protocol, however, generated high titre intestinal and serum IgA and IgG responses to CT and TG which were similar to those generated by mucosal immunization with CT and unconjugated TG (Table 2).

Parenteral immunization generates high titre serum IgG anti-AFB

To test whether the rabbits were capable of mounting a systemic response to the AFB determinant, animals were immunized i.m. with AFB–TG in CFA followed 4 weeks later by a booster immunization with AFB–TG in IFA (Table I, group VI). This immunization protocol generated high titre serum IgG anti-AFB and anti-TG responses which were comparable with those generated by immunization with unconjugated TG in CFA/IFA (Table I, group VII) (Table 2).

Mucosal unresponsiveness is not accompanied by systemic 'oral' tolerance

As the AFB determinant appeared to be refractory to the adjuvancy of CT, it was of interest to know whether immunization with the conjugate preparation was associated with a concomitant systemic tolerance ('oral tolerance') to this determinant. A group of rabbits was immunized weekly via ileal loops with AFB–TG plus CT followed by an i.m. immunization with AFB–TG in CFA on day 25,

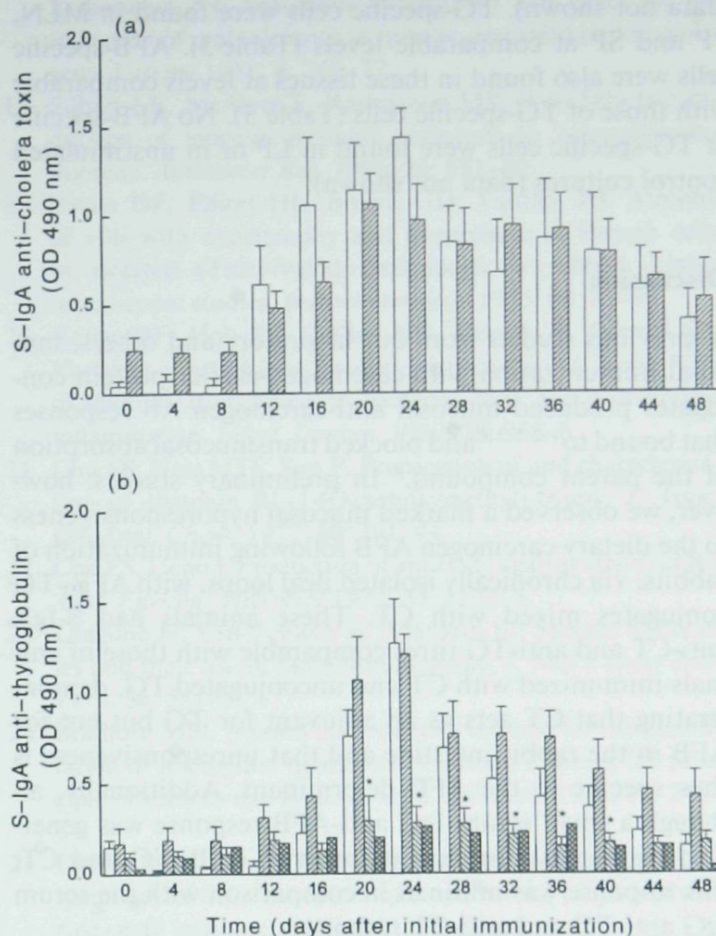

Figure 2 Mucosal S-IgA anti-cholera toxin (CT) and anti-thyroglobulin (TG) responses. Animals were primed (a) with (□) 100 µg CT + 100 µg of aflatoxin B_1–thyroglobulin (AFB–TG) or (▨) 100 µg TG + 100 µg of CT; and (b) with (□) 100 µg CT + 100µg AFB–TG; (▩) 100 µg of TG alone; (▧) 100 µg AFB–TG alone; or (▨) 100 µg of TG + 100 µg of CT, via chronically isolated ileal loops, on day 0 as indicated, and given identical booster immunizations on days 7, 14 and 21. The data points indicate the mean response (SEM). Intestinal secretions at 4-day intervals were assayed at a dilution of (a) $1:2^{11}$ or (b) $1:2^{10}$ and measured by amplified ELISA; $n = 5$ for AFB–TG with CT, TG with CT, and AFB–TG groups; $n = 2$ for TG alone group. Asterisks in panel (b) denote a significant difference ($P < 0.05$) from the group immunized with AFB–TG mixed with CT.

and an i.m. booster immunization with AFB–TG in IFA on day 55 (Table 1, group V). This immunization protocol generated high titre serum IgG responses to AFB and TG, which were comparable with those generated by i.m. immunization with AFB–TG in CFA–IFA without prior immunization via ileal loop (Table 2).

AFB-specific B cells reside in mucosal lymphoid tissue

In order to determine whether the inability of the mucosal arm of the immune system to mount an anti-AFB response was caused by a lack of AFB-specific B-cells in mucosal lymphoid tissue, we stimulated lymphocytes from LP, MLN, PP, and SP of unimmunized animals with the mitogens LPS and PWM. This method was chosen because *in vitro* stimulation of PP and SP lymphocytes from naive rabbits with AFB–TG proved unsuccessful

(data not shown). TG-specific cells were found in MLN, PP and SP at comparable levels (Table 3). AFB-specific cells were also found in these tissues at levels comparable with those of TG-specific cells (Table 3). No AFB-specific or TG-specific cells were found in LP or in unstimulated control cultures (data not shown).

Discussion

In previous studies from our laboratory and others, mucosal immunization with carcinogen-carrier protein conjugates produced mucosal anti-carcinogen Ab responses that bound to[1,2,5,18] and blocked transmucosal absorption of the parent compound.[4] In preliminary studies, however, we observed a marked mucosal hyporesponsiveness to the dietary carcinogen AFB following immunization of rabbits, via chronically isolated ileal loops, with AFB–TG conjugates mixed with CT. These animals had S-IgA anti-CT and anti-TG titres comparable with those of animals immunized with CT and unconjugated TG, demonstrating that CT acts as an adjuvant for TG but not for AFB in the rabbit intestine and that unresponsiveness is thus specific to the AFB determinant. Additionally, although a small serum IgG anti-AFB response was generated by mucosal immunization with AFB–TG and CT, this response was minimal in comparison with the serum IgG anti-TG and anti-CT responses.

Parenteral immunization with AFB–TG in CFA/IFA generated a strong serum IgG anti-AFB response that was comparable in titre to the serum IgG anti-TG response. Thus we infer that unresponsivess to AFB was restricted to the mucosal arm of the immune system. These experiments also demonstrate that the conjugate preparation presented the AFB determinant in an immunogenic form and that the rabbits possess the requisite systemic B and T cell repertoires to mount an anti-AFB response. Furthermore, the serum IgG anti-AFB and anti-TG responses of animals immunized mucosally with AFB–TG and CT prior to systemic immunization with AFB–TG in CFA–IFA were not significantly different from those of animals immunized with AFB–TG or TG alone in CFA–IFA without prior mucosal immunization. These observations demonstrate that mucosal unresponsiveness to AFB is not accompanied by systemic tolerance and is thus a distinct phenomenon from 'oral tolerance.' It is possible, however, that CFA provides a stronger immunogenic signal than CT and is thus able to overcome any systemic tolerance that may have developed.

Mitogen stimulation of cells isolated from PP, SP and MLN of unimmunized animals revealed the presence of AFB- and TG-specific ASC at comparable levels in both systemic and mucosal lymphoid tissues. Although this method did not allow a quantitative estimate of the precursor frequency of AFB-specific B cells in these tissues, it does demonstrate that B cells that were functionally anergic *in vivo* could be activated *in vitro*, in agreement with the findings of Moller.[28]

Although active suppression to AFB was not demonstrated in these studies, the following lines of evidence support the notion that unresponsiveness to AFB is maintained in the rabbit mucosa. First, AFB-specific B cells are present in the mucosal lymphoid tissues, but do not respond to antigen stimulation, even when the antigen is co-administered with CT. This mucosal adjuvant can generate Ab responses to co-administered Ag that are as much as 10- to 1000-fold stronger than those generated by Ag alone (Table 2,[6,18]). Second, in previous studies, AAF-carrier protein conjugates administered to rabbit ileal loops at the same dose as was used in the present study generated strong S-IgA anti-AAF responses.[4–6,19]

It is unclear how specific unresponsiveness to AFB is established or maintained in the milieu of a vigorous, Th2 dominated response to both CT and to TG, the latter of which was covalently coupled to AFB. Our observations, nonetheless, demonstrate that unresponsiveness to AFB is hapten-specific, restricted to the mucosal immune system, and refractory to the adjuvancy of CT.

Table 3 Spot forming cells to aflatoxin B_1 and thyroglobulin from lymphoid organs of unimmunized animals

Organ	Spot forming cells
aflatoxin B_1	
Peyer's patches (*n* = 4)	18 ± 3
Spleen (*n* = 4)	11 ± 4
Mesenteric lymph nodes (*n* = 3)	17 ± 1
thyroglobulin	
Peyer's patches (*n* = 4)	8 ± 2
Spleen (*n* = 4)	11 ± 3
Mesenteric lymph nodes (*n* = 3)	3 ± 2

Data represent the number of antibody secreting cells (ASC) per 10^6 stimulated lymphocytes (mean ± SE) after background subtraction. The numbers of antibody secreting cells to aflatoxin B_1 were not significantly different from those to thyroglubulin in corresponding organs by Student's *t*-test.

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