Binding reaction of aflatoxin B_1 with immunoglobulin G against aflatoxin B_1-bovine serum albumin complex

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Summary
Polyclonal immunoglobulin G antibodies were raised against aflatoxin B_1-bovine serum albumin complex and characterised. Antibodies against the complex were obtained after a single intradermal multiple site injection of water in oil emulsion of the complex into adult female albino rabbits. Equilibrium dialysis and Scatchard plot analysis of the interaction of the antibodies with aflatoxin B_1 showed that the antibodies have high affinity for binding aflatoxin B_1. The average number of binding sites on the antibody molecules for aflatoxin B_1 is 1.74 ± 0.20 with mean standard free energy change ($\Delta F^0{}_1$) of -23.10 KJ/mol, while the average association constant is 2.35 ± 0.73 x $10^{-4}M^{-1}$. Male wistar strain albino rats after immunization with the complex showed lower mortality when challenged with a single dose of aflatoxin B_1. The results suggest that such antibodies with high affinity for aflatoxin B_1 could be used in the immunointerception of the toxin.

Keywords: *Aflatoxin B_1 (AFB_1), Aflatoxin B_1-bovine serum albumin (AFB_1 -BSA), immunoglobulin G (IgG), immunointerception.*

Résumé
Anticorps polieclonale immunoglobuline G était comparé avec l'aflatoxin B_1-le bouvine sérum alboumine complexe et caracterise. L'anticorps contre la complexe était obtenu après un seul intradermal multiple piqure de l'eau à l'huile d'émulsion de la complexe aux adultes lapins de type albino. L'étude dialysis equilibre et d'une analyse d'un endroit de l'interaction de l'anticorps avec l'aflatoxin B_1, a montré que l'anticorps a une affinité de niveau élévé pour l'aflatoxin B_1. Le numéro moyen de contraignant liéà la molécule de l'anticoprs pur l'aflatoxin B_1 est 1.74 ± 0.20 avec moyen standard free energy change (($\Delta F^0{}_1$) de -23.10 KJ/mol, loraque l'association constant moyen est 2.35 ± 0.73 x 10^{-4} M^{-1}. Après l'immunisation des rats de type wistar avec le complexe, ceci a montré une biasse de la mortalité quand on a défié avec une seule dose d'aflatoxin B_1. Les résultats suggèrent que tels anticoprs avec une hausse attraction pour l'aflatoxin B_1 utilisé dans l'immunointerception de la toxine.

Introduction

Aflatoxin B_1 (AFB_1) is one of the potent chemical carcinogens occurring as natural products and contaminants of human foodstuffs [1]. The populace in tropical countries including Nigeria are thus inadvertently exposed to this toxin. Aflatoxin B_1 has been shown to induce varying degrees of toxicities and liver tumours in animals [2,3]. It has also been implicated in the etiology of primary liver cancer and some other tumours in man [4]. Because of the potential health hazard constituted by this toxin, various attempts are being made towards determining the biochemical and molecular mechanisms underling aflatoxin toxicogenesis and carcinogenesis in man and animals [5].

In the last two decades, research work has centred around the production of polyclonal and monoclonal antibodies against protein and DNA adducts of AFB_1, specifically AFB_1-N_7-guanine and AFB_1-serum albumin adducts [6,7]. These antibodies have been used extensively in immunoassays to detect aflatoxins in food samples [8]. They have also been used to screen human populations at high or low risk of exposure to AFB_1 on the basis of the presence of the toxin or its metabolites in their urine and serum samples [9,10].

Animals immunized with carcinogens complexed to proteins especially albumin have been shown to form antibodies that recognize the carcinogen [11]. In order to understand the relevance of the interaction between AFB_1 and antibodies against AFB_1-bovine serum albumin in protection against cancer, we have raised polyclonal antibodies against the complex and studied the binding parameters of its immunoglobuline G antibodies with AFB_1. We also studied the effect of immunizing rats with complex on the toxicity of aflatoxin B_1.

Materials and Methods

Aflatoxin B_1 and Aflatoxin B_1 buvine serum albumin complex were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Visking cellophane dialysis tubing was purchased from Scientific Instrument Centre, London. Before use, the tubings were cleaned by boiling thrice in EDTA-distilled water solution. They were stored in 20ml distilled water containing 0.2 ml of 0.2M EDTA solution at 4°C.

Experimental animals A total of eight adult female albino rabbits and forty-five matured male wistar rats were obtained from the Central Animal House, College of Medicine, University of Ibadan, Ibadan and used for the study.

Immunization of rabbits The multiple site intadermal injection method of Chu and Ueno [12] was employed. Essentially 1 ml Aflatoxin B_1-bovine serum albumin was dissolved in 5 ml 0.90% NaCl. The antigen solution was mixed with 10 ml complete Freund's adjuvant and additional 10 mg Mycobacterium tuberculosis to give a water in oil emulsion with final concentration of 66.67 µg protein for ml of emulsion. After this, 2 ml each of this emulsion was injected separately into four of the adult female rabbits intradermally at 20 to 40 sites along the shaved area of their back. Again, 2 ml each of saline was injected intradermally into the remaining four rabbit as controls. Trial bleedings were made via the inner marginal ear vein once every week starting from the third week after immunization to the eighth week after immunization.

Approximately, 25 ml blood samples were collected weekly from each of the test and control rabbits and separately treated as follows: For each week, serum was

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prepared from each of the freshly drawn blood samples. Presence of antibodies to AFB_1-BSA complex was detected in the serum by the interfacial test in which a solution of the antiserum was carefully overlaid with that of the complex, precipitate formation was then examined around the interface. Equal volumes of the antisera from the four (4) test rabbits were mixed together to make the pool of test antiserum for that week. Sera from the control rabbits were similarly treated to get the pool of control serum for the week.

Preparation of immunoglobin G (IgG) antibodies: Imunoglobulin G was prepared from each of the pooled antisera and control sera by three precipitation with saturated ammonium sulphate solution at a final concentration of 33.3%. The third precipitate was dissolved and dialysed against borate buffered saline and finally against sodium phosphate buffer (NaPB) at pH 6.3. It was further purified by chromatogrpahy on a diethylaminoethyl cellulose column, which was equilibrated with NaPB at pH 6.3. IgG was eluted from the column with the same buffer (Fig. 1), pooled, concentrated and stored frozen for subsequent tests. Immunoglobulin concentration (in the weekly samples) was determined spectrophotometrically at 278 nm using an absorptivity ($1^{1\%}{}_{1cm}$) of 15.5.

Determination of binding parameters of the antibodies for aflatoxin B_1. The equilibrium dialysis method was used to determine the specificity of the antibodies obtained from the immunized rabbits. Aflatoxin B_1 solution was prepared in borate buffered saline in a concentration range of 50, 20, 10, 5 and 1 uM, respectively. The dialysis tubing holding 0.5 ml antibody globulin from each antiserum sample was placed in the appropriate aflatoxin B_1 solution. The bottles containing the solution were capped tightly, wrapped with foil paper and refrigerated for 24 hours to allow for equilibration. Controls consisting of 0.5 ml globulin from control rabbits were similarly treated. The amount of free aflatoxin in equilibrium with bound aflatoxin was estimated by measuring the concentration spectrophotometrically at 363 nm. The amount of IgG bound aflatoxin B_1 was calculated by subtracting the concentration of free and unbound toxin from the initial concentration. The moles of aflatoxin B_1 bound per molecule of control globulin was subtracted from the mole of the toxin bound per molecule of test globulin to correct for non-specific binding. The binding constant and the number of binding sites were analysed using the scatchard equation [13] assuming the law of mass action.

Immunization of rats: Male wistar rats each weighing approximately 200 g were divided into three groups of 15 rats each. Rats in one of the groups (A) were immunized subcutaneously with aflatoxin B_1-bovine serum albumin complex in normal saline (100 μg protein/rat). Rats in the remaining two groups (B and C) were injected with normal saline. Three weeks after immunization, presence of antibodies in the serum of the immunized rats was detected by the interfacial test. The rats in groups A and B were then challenged with a single dose of pure aflatoxin B_1 in dimethylsulphoxide (DMSO) (7 mg/kg body wt) by interperitoneal injection. Mortality was monitored.

Results and Discussion

Exposure of animals and man to environmental compounds has been shown to cause diverse reactions in vivo. Such reactions include immunostimulations, autoimmune reactions and immunologic responses resulting in the formation of antibodies against hapten conjugates [14]. In the present study, antibodies against the AFB_1-BSA conjugate were demonstrated in all the antisera from the immunized animals. All the antisera were purified by repeated ammonium sulphate precipitation followed by chromatography on diethylaminoethyl cellulose to give immunoglobulin samples free of serum albumin. Contamination by albumin may lead to false positive results due to the high affinity of AFB_1 for the protein [15].

Optimal production of antibodies seems to occur in the seventh week after immunization (Figs 1 & 2).

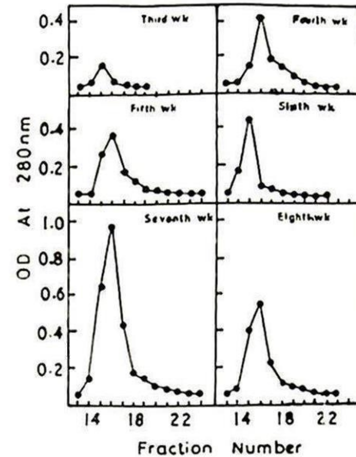

Fig. 1 Elution profile of exchange chromatograph on DEAE-cellulose of the isolated rabbit immunoglobin G antibodies, starting from the third week to the eighth week after immunization

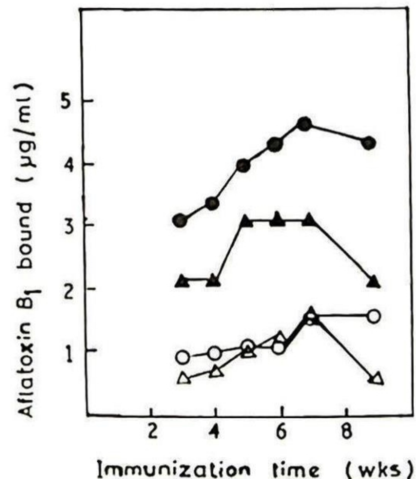

Fig. 2 Production of antibodies against aflatoxin B_1 and concentration dependent binding of aflatoxin B_1 to the antibodies The different concentrations of aflatoxin B_1 used are 34 9 (●), 18 4 (▲), 9.67 (Δ) and 5.93 (O) μg/ml, respectively

The antibody titers in figure 2 showed that for each of the five different concentrations of AFB_1 used in the binding studies, maximum binding of the toxin occur with the antibodies purified from the antiserum obtained in the seventh week. Scatchard plot analysis of the binding measurements made for each of the six weekly antisera samples (Table 1; Fig. 3) suggests that the average number of binding sites per antibody molecule for AFB_1 is 1.74 ± 0.20. This agrees within the limits of experimental error with observations made earlier for a molecule of a precipitating antibody [16, 17]. The immunoglobulin G antibodies may therefore be precipitating antibodies.

Table 1: Binding data of aflatoxin B_1 to IgG antibody

Week after Immunization	Number of binding sites (n)	Association constant $K_1M^{-1} \times 10^{-4}$	Standard free energy change (ΔF^0_1) KJ/mol
3	1.90	3.63	-24.18
4	1.74	2.68	-23.47
5	1.85	2.36	-23.18
6	1.96	1.91	-22.72
7	1.46	1.94	-22.76
9	1.54	1.59	-22.10
Mean	1.74 ± 0.20	2.35 ± 0.73	-23.10

The values of n and K_1 were obtained from scatchard plots of the binding data. The standard free energy change for the first ligand bound (ΔF^0_1) was calculated from K_1 by the general thermodynamic equation $\Delta F^0_1 = RTInk_1$. R is the gas law constant and T is the absolute temperature. ΔF^0_1 was expressed as KJ/Mol (1 cal. = 4.184J)

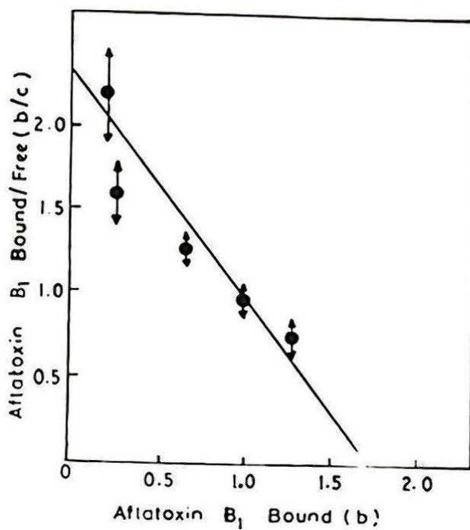

Fig 3 Scatchard plot of data for binding of aflatoxin B_1 to immunoglobulin G antibodies. Scatchard equation for the law of mass action, b/c = Kn-Kb was made use of, where b is the molar ratio of bound AFB_1 to antibodies; c is the molar concentration of the free AFB_1 at equilibrium; n is the average number of binding sites on an antibody and K is the average association constant for the binding at each site. Each point on the graph is the mean of six determinations for the six weeks under study. The intercept on the ordinate is K (the association constant for the first ligand bound) while the one on the abscissa is n (the average number of binding sites on each antibody)

The mean association constant of $2.35 \pm 0.73 \times 10^{-4}$ M^{-1} for the binding indicates high affinity of the antibodies for aflatoxin. This suggest the possible utilization of the antibodies as immunodetectionve or immunointerceptive agents for the toxin. Absorption of aflatoxin B_1 from the gastrointestinal tract after ingestion has been shown to be followed by its immediate transport to the liver for metabolic activation [18]. Since dietary exposure to aflatoxins cannot be prevented for now in developing countries, a possible mechanism of reducing its effect may be by blocking its transport to and consequent activation in the target organs. Although aflatoxin B_1 is a hapten, a humoral immune response may be elicited in humans on exposure to the toxin [19]. This response could be due to aflatoxin conjugation with serum albumin during its transport. It has been shown that the intake of aflatoxin B_1 correlates with the level of aflatoxin-albumin conjugate in the peripheral blood [120]. Therefore, active immunization of animals with aflatoxin B_1-bovine serum albumin could result in the production of sufficient IgG antibodies with high affinity for the toxin. The antibodies will most likely inhibit aflatoxin transport and activation.

The protective effect of immunization has been demonstrated by the mortality rate observed in the unimmunized rats challenged with aflatoxin B_1 as compared with the immunized rats (Table 2). After the rats were immunized and challenged with the mycotoxin, the serum IgG antibodies produced in the immunized rats will probably remove aflatoxin B_1 from the system. Since metabolic activation plays an important role in aflatoxin B_1 toxicity, interaction of the toxin with the antibody will prevent its conversion to aflatoxin B_1-2, 3-epoxide, the ultimate carcinogen, thus inhibiting the covalent interactions with cellular macromolecules and the consequent toxic effects. Therefore, it may be possible to immunointercept aflatoxin B_1, but it is not known whether this might protect against aflatoxin B_1 induced hepatocarcinogenesis.

Table 2: Mortality rate in immunized and unimmunized rats exposed to aflatoxin B_1

	Immunized	Unimmunized controls	
		Positive	Negative
Groups	A	B	C
AFB_1-BSA (μ protein/rat)	100	0	0
Saline (ml)	0	1.0	1.0
AFB_1 (mg/kg bd. Wt.)	7	7	0
Mortality (No. of deaths/surviving)	3/15	11/15	0/15

Mortality was recorded in 1-5 days after a single dose or interperitoneal injection with pure aflatoxin B_1.

Additional studies are therefore required to understand the interrelationship of aflatoxin B_1 and the IgG antibodies and the long-term effect of such interaction on aflatoxin B_1 induced hepatocarcinogenic effects.

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