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Immune response to aflatoxin B_1-histone H_1 Complex

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Summary
Antibodies against aflatoxin B_1 have been raised in rabbits using aflatoxin B_1-histone H_1 conjugates as immunogen. Aflatoxin B_1 was coupled to histone H_1 via the ultimate carcinogen aflatoxin B_1-2, 3-epoxide. The antibodies are specific for aflatoxin B_1. The average number of binding sites on the antibody molecules for aflatoxin B_1 as obtained from Scatchard plot analysis of the binding data is 1.94 with $<F^\circ$ = -6.19 Kcal/mol, while the average association constant for the binding is 34.5 x 10^3 M^{-1}. Male wistar rats after immunization with aflatoxin B_1-histone H_1 showed lower mortality and reduction of acute toxic effects in the liver when challenged with a single dose of aflatoxin B_1.The antibodies may be useful in immunoprophylaxis against aflatoxicosis.

Keywords: *Aflatoxin B_1-histone H_1-complex, immunoprophylaxis.*

Résumé
On eleve des anti-corps dans les lapins contre l aflatoxine B_1 en utilisant comme immunogene la combinaison aflatoxine B_1-histone H_1. On avait accouple l aflatoxine B_1 et l histone H_1 a travers l ultime carcinogene aflatoxine B_1-2, 3-epoxide. Les anti-corps sont specifiquement destines a l aflatoxine B_1. Le nombre moyen de sites contraignants sur les molecules d anti-corps pour l aflatoxine B_1, obtenu de l analyse du terrain de scatchard sur les donnees de combinaison, est de l, 94 avec $<F^\circ$ = -6,19Kcal/mol, tandis la constante d association moyenne pour l element de combinaison est de 34,5 x 10^3M^{-1}. Les rats wistars males, apres avoir ete vaccines avec la combinaison de l aflatoxine B_1-histone H_1, faisaient preuve de mortalite moins frequente et de toxicite moins aigue dans leurs foies sous l impact d une seule dose de l aflatoxine B_1. Les anti-corps pourraient servir d immunoprophylaxie contre l aflatoxicose.

Introduction

The cytotoxicity, mutagenicity and carcinogenicity of aflatoxin B_1 (AFB_1) in various cells have been well-documented [1-3]. This mycotoxin has been shown to interact both *in vivo* and *in vitro* after metabolic activation with serum albumin [4] and target cell nuclear DNA especially within the rRNA gene sequences [5]. In addition to DNA and RNA's [6], AFB_1 also interact with nuclear proteins especially the lysine rich histone H_1 protein [7]. Antibodies raised against DNA and serum albumin adducts of aflatoxins are being used in the immunodetection and immunodiagnosis of these toxins [8-10]. It was therefore thought desirable to assess the immunological potentials of aflatoxin B_1-histone H_1 (AFB_1-H_1) complex.

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Furthermore, the use of macromolecular carriers such as DNA, serum albumin and histone H_1 which are *in vivo* targets of AFB_1 in raising antibodies against the toxin may give an insight into possible immune response to such complexes that may be formed during the toxins' biochemical interactions with the carriers *in vivo*. Such knowledge is likely to reveal their potential use in immunoprophylaxis against aflatoxins.

We report here the coupling of AFB_1 to histone H_1, characterisation of antibodies to the complex and the protective effect of the antibodies on AFB_1 -induced hepatic toxicities.

Materials and methods

Aflatoxin B_1, histone H_1, nicotinamide adenine diphosphate (NADP), glucose-6-phosphate (G6P) and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Aroclor 1254 was from Monsanto, Co. (St. Louis. Mo.). Sephadex G-100 was purchased from Pharmacia Fine Chemical, Inc., while cellulose acetate strips were from Gelman Instrument Co. Complete Freund's adjuvant and *Mycobacterium tuberculosis* (H37Ra) were obtained from Difco Laboratories (Detroit, M.I.). Male albino rabbits of approximately 2 kg size and male albino wistar rats approximately 200 g size were obtained from the Central Animal House, College of Medicine, University of Ibadan, Ibadan, Nigeria. All chemicals and organic solvents were either reagent grade or chemically pure.

Preparation of rat liver homogenate fraction (S_9)

The rat liver microsomal fraction (S_9) (a postmitochondrial supernatant at 9,000 g) was prepared according to the method of Maron and Ames, 1983 [11]. Male albino wistar rats weighing approximately 200 g each were used. The rat liver enzymes were first induced with Aroclor 1254 (a polychlorinated biphenyl (PCB) mixture). Aroclor 1254 was diluted in corn oil to a concentration of 200 mg/ml and a single intraperitoneal (i.p.) injection of 500 mg/kg was administered to each of the rat five days before sacrifice. The rats were given rat cubes (from Ladokun Livestock Feeds, Ibadan, Nigeria) and drinking water ad libitum until 12 hours before sacrifice when the food alone was removed.

All the steps involved in the preparation of the S_9 fraction were carried out at 0-4 °C using cold, sterile solutions and glasswares. On the fifth day of induction, the rats were killed by cervical dislocation and their livers excised aseptically using sterile surgical tools. The freshly excised livers were placed in preweighed beakers containing approximately 1 ml of chilled 0.15M KCl/g of wet liver. After weighing, the livers were washed several times in fresh, chilled KCl, transferred to

a beaker containing 3 volumes of 0.15M KCl (3 ml/g wet liver) and homogenized. The homogenate was centrifuged at 9,000 g for 15 minutes at 4 °C in the MSE 18 refrigerated centrifuge. The supernatant (the S_9 fraction) was decanted, sterilized by filtration through a 0.22 mμ filter under positive pressure, distributed in 2 ml portions in small plastic Nunc tubes and stored immediately at -80 °C.

The sterility of the preparation was determined by plating 0.1 ml of the S_9 homogenates on minimal agar containing histidine and biotin.

Preparation of immunogen

The immunogen aflatoxin B_1-histone H_1 (AFB_1-H_1), was prepared by a modification of the methods of Gurtoo, [12] and Essigmann *et al.*_[3]. The incubation mixture for the conjugation of AFB_1 to histone H_1 included sodium phosphate buffer (NAPB) (pH 7.4; 100 mM); $MgCl_2$ (8 mM); KCl (33 mM); G6P (5 mM); NADP (4 mM); approximately lmg of S_9 microsomal proteins (prepared as described above) per ml of 1% w/v histone H_1 in NAPB and AFB_1 in DMSO. The molar ratio of hapten to carrier protein in the reaction mixture was 25. The reaction mixture was agitated at 37 °C for 90-minutes in complete darkness. The mixture was subsequently dialyzed against seven changes of distilled water in the dark at 0 °C to 4 °C for 24 hours to remove the free hapten AFB_1.

The crude suspension of AFB_1-H_1 complex was further purified on sephadex G-100 column. Eluate from the column was concentrated by vacuum dialysis at 0 °C-4 °C. The concentrated suspension of AFB_1-H_1 was distributed into evaporation dishes and allowed to dry under vacuum in a desicator at 0 °C - 4 °C.

The above procedure was repeated except that the S_9 microsomal proteins were denatured at 100 °C for 10 mins before being added to the reaction mixture followed by AFB_1.

The dried crystals from each of the reaction medium were separately pooled and stored desicated in amber coloured bottles at 4 °C. Samples from the two stock crystals were analysed for covalent binding between AFB_1 and H_1 and for immunochemical properties.

Solvent extraction

One percent aqueous solution of samples of AFB_1-H_1 complex from the first and second mixtures were separately extracted with 10 ml chloroform for 10mins and were allowed to stand in the dark for 30mins during which the aqueous and chloroform layers separated. The two layers
were subsequently examined under the long wavelength ultraviolet light for the presence of the blue fluorescence characteristic of AFB_1.

Electrophoresis

Samples from three (0.75%, 3%, 15%) aqueous solutions of AFB_1-H_1 complex from the first reaction medium were run on two cellulose acetate strips at 0.4mA per centimeter of strip width for two hours in Barbital buffer (pH 8.6 0.1). After electrophoresis one of the strips was stained with Ponceau S while the unstained strip was observed under the long wavelength ultraviolet light for fluorescence (Figure 1).

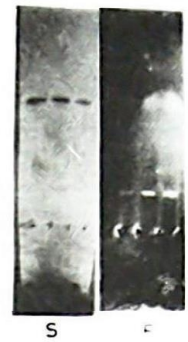

Fig.1: Cellulose acetate electropherogram of aflatoxin B_1-histone H_1 complex. Three dilution of AFB_1-H_1 aqueous solution (0.75%; 3%; 15%) were run on the two cellulose acetate strips at 0.4mA per centimeter of strip width in Barbital buffer (pH 8.6 0.1) for 2 hours. S:- stained with Ponceau S, F:- unstained and photographed under long wavelength ultra violet light.

Spectrophotometric analysis

The UV spectra of AFB_1-H_1 complex from the first reaction medium, pure AFB_1 and pure histone H_1 were obtained in saline for comparison (Figure 2).

The amount of AFB_1 bound to histone H_1 in the complex obtained from the first reaction mixture was determined spectrophotometrically after the conjugate had been extracted with chloroform [14]. Data obtained from both the unreacted toxin or the toxin conjugated to protein were used for final calculation.

Production of antibodies against AFB_1-H_1

Antibodies to AFB_1-H_1 complex were raised in three rabbits after they had been separately injected intradermally with 2 ml of water in oil emulsion of the complex (66.67) μg protein/ml) at 20 to 40 sites along the shaved area of their back [15]. The water in oil emulsion was prepared by dissolving 1 mg AFB_1-H_1 in 5 ml 0.9% NaCl. 10ml complete Freund's adjuvant and additional 10mg *Mycobacterium tuberculosis* were added to the solution to give a water in oil emulsion with final concentration of 66.67 ug protein

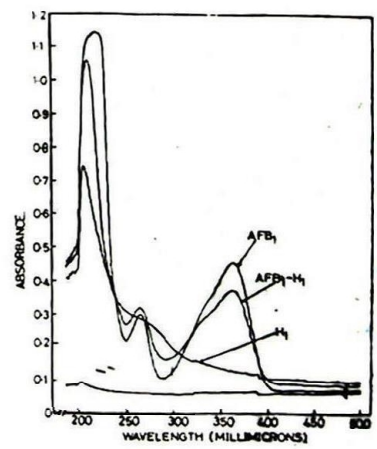

Fig. 2: UV spectra of aflatoxin B_1 (AFB_1); aflatoxin B_1-histone H_1 complex (AFB_1-H_1) and histone H_1 (H_1) in saline.

per ml of emulsion. 2 ml of saline was injected into three control rabbits. Trial bleedings were made through the inner marginal vein of each of the rabbit's right ear once every week starting from the third week to the twelfth week after immunization. For each week, the presence of serum antibodies to AFB_1-H_1 was detected by the interfacial ring test where a solution of the antiserum was carefully overlaid with that of the complex. Precipitate formation was then examined around the interface. The antibody titers of the immunized rabbits were also determined on a weekly basis (Fig. 3).

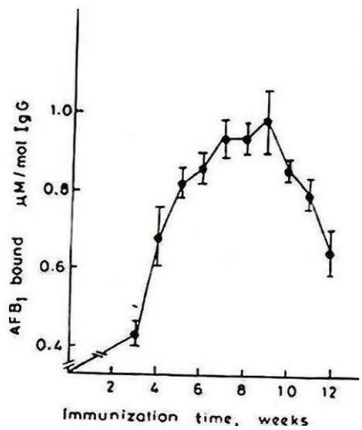

Fig. 3: Antibody titres of the rabbits after immunization with aflatoxin B_1-histone H_1 complex. Each point on the graph is the mean of the data obtained for the three rabbits in the ten weeks under study.

Immunoglobulin G (IgG) was prepared and purified from the weekly antisera and control sera as follows: - IgG was separated from the serum samples by three precipitations 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 chromatography on a diethylaminoethyl cellulose column, which had been equilibrated with NaPB at pH 6.3. IgG was eluted from the column with the same buffer, pooled, concentrated and stored frozen for subsequent tests. Immunoglobulin concentration in the weekly samples was determined spectrophotometrically at 278 nm using an absorptivity ($E^{1\%}$) of 15.5. The specificity of the IgG antibodies for AFB_1 was determined using the hapten inhibition reaction [16]. Figure 4 shows the inhibition curve for the reaction.

Physicochemical characterisation of the antibodies against AFB_1-H_1 complex.

Equilibrium dialysis method was utilized to study the binding properties of the antibodies from the immunized rabbits with AFB_1. AFB_1 solution was prepared in borate buffered saline in a concentration range of 50, 20, 10, 5 and 1uM respectively. The dialysis tubing holding 0.5 ml antibody globulin from each antiserum sample was placed in the appropriate AFB_1 solution. The solutions were refrigerated in the dark 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 spectrophotometrically at 363 nm. The amount of IgG bound AFB_1 was determined as earlier described [17]. The binding constant and the number of binding sites were analysed using the Scatchard equation [18] assuming the law of mass action. (Fig. 5).

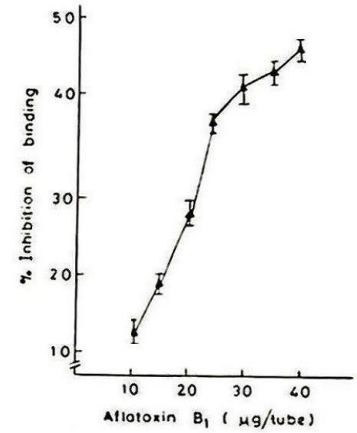

Fig. 4: Inhibition of binding of rabbit immunoglobulin G antibodies to aflatoxin B_1-histone H_1 complex in the presence of aflatoxin B_1

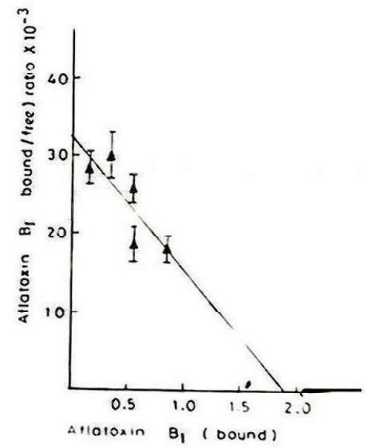

Fig. 5: Scatchard plot of data for binding of aflatoxin B1 to rabbit anti-aflatoxin B_1-histone H_1 antibodies. Scatchard equation for the law of mass action b/c = kn-kb was made use of:
Where b is the molar ratio of bound aflatoxin B_1 to antibodies; c is the molar concentration of free aflatoxin B_1 at equilibrium; n is the average association constant for the binding at each site. Each point on the graph is the mean of the data obtained for the ten weeks understudy. 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 molecule).

Determination of the protective effect of the antibodies on AFB_1-induced hepatic toxicities

The male wistar rats were divided into three groups (I, II, III) of twenty-five rats each. Rats in group I were immunized

intradermally with water in oil emulsion of AFB_1-H_1 complex (100 ug protein/kg body wt. of rat) while those in groups II and III had intradermal injection of saline. The animals were kept for four weeks after which the antibody titers of the immunized rats were determined. All the animals in groups I and II were then challenged with a single dose of pure AFB_1 in DMSO (7 mg/kg bd. wt.) by intraperitoneal injection and the mortality rate was monitored. The surviving rats were kept for two weeks after which they were bled via cardiac puncture and their livers asceptically excised.

1 g of the liver sample from each of the rats was separately homogenized in normal saline (3 volumes of liver wt.) and the protein content of the supernatant obtained on centrifugation (3,000 rpm for 15 mins) determined. Serum samples and supernatant from the liver homogenates (7.65 mg protein/ml) were analysed for alkaline phosphatase (ALP) and gamma glutamyl transferase (GT) activities. (Fig. 6). Limited histological examinations were also performed on the livers of the dead and surviving rats.

Results

AFB_1 was complexed to histone H_1 in the presence of S_9 microsomal activation mixture. The blue fluorescence in ultraviolet light characteristic of AFB_1 remained in the aqueous solution layer of AFB_1-H_1 complex from the first reaction medium after 10 minutes of vortexing with chloroform. The fluorescence was however extracted into the chloroform layer when the complex obtained from the second reaction medium in which the S_9 microsomal proteins were denatured before being added to the reaction mixture was similarly treated.

The electropherogram of AFB_1-H_1 complex (Fig.1) shows that the protein spots stained with Ponseau S correspond with the fluorescence spots of unstained duplicate electropherogram photographed under the long wavelength ultraviolet light.

The UV spectra in Figure 2 revealed that there was a red or bathochromic shift in the spectrum for AFB_1-H_1 and a blue or hypsochromic shift in the one for histone H_1 vis-à-vis the spectrum for AFB_1.

Antibodies were produced against AFB_1-H_1 complex in all the weeks studied with maximum production occurring in the ninth week after immunization. (Fig.3). The antibodies are specific for AFB_1 as demonstrated from the inhibition curve (Fig. 4). The average number of binding sites on the antibodies for AFB_1 is 1.94 with mean classical association constant of $34.5 \times 10^3\ M^{-1}$ and standard free energy change of -6.19 Kcal/mol. (Fig. 5).

The immunoprotective potential of the antibodies raised against AFB_1-H_1 complex was demonstrated by high mortality rate of the unimmunized rats and the reduced mean serum and liver ALP and GT activities in immunized rats compared to their unimmunized counterparts (Fig. 6) coupled with the reduction of AFB_1 induced hepatic lesions observed in the livers of rats immunized with AFB_1-H_1 complex before challenge with AFB_1.

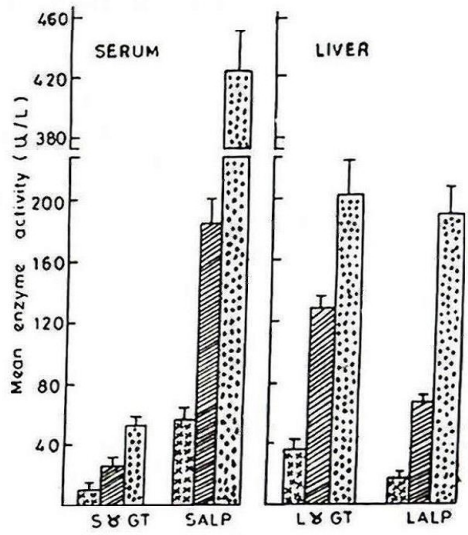

Fig. 6: Effect of immunization with aflatoxin B_1-histone H_1 complex on serum and liver gamma glutamyl transferase and alkaline phosphatase in rats exposed to aflatoxin B_1

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***** control, unimmunized and unchallenged rats.

/////// Rats immunized with aflatoxin B_1-histone H_1 complex followed by challenge with aflatoxin B_1

!!!!! Unimmunized rats challenged with aflatoxin B_1

Discussion

Earlier findings had shown that AFB_1 bind to histone H_1 (7) and that such binding may alters chromatin structure/function and gene expression *in vivo* (19). Our data presented on the *in vitro* coupling of AFB_1 to histone H_1 in the presence of S_9 activation mixture suggests that the bond between AFB_1 and histone H_1 in the synthesized complex is probably covalent. Such covalent bonds cannot be broken by solvent extraction of AFB_1-carrier molecules with chloroform. The electropherogram of AFB_1-H_1 in Figure 1 also support this observation. The UV spectra in Figure 2 clearly suggests that there has been a coupling of AFB_1 to histone H_1. Thus, this *in vitro* binding of AFB_1 to histone H_1 seem to have occur via the ultimate carcinogen AFB_1-2, 3-epoxide reacting directly with the episilon amino group of the lysyl amino acid residues in the lysine rich histone H_1. Lysine had been shown to be the amino acid preferentially targeted by AFB_1 in proteins [20].

Animals immunized with carcinogen conjugated to proteins including AFB_1-protein conjugates have been shown to form antibodies that recognise the carcinogen [8, 21]. In this study, the specificity of antibodies to AFB_1-H_1 complex (Fig. 3) for AFB_1 was established from the fact that AFB_1 inhibited the binding of the antibodies to AFB_1-H_1 complex that induced their formation (Fig. 4). The mechanism of this inhibition seems to involve the formation of soluble complexes between the IgG antibodies and AFB_1 molecules thereby preventing the combination of the antibodies with the larger complete molecule of antigen which induce the formation of visible antigen-antibody complexes.

The average number of binding sites of 1.94 on the antibodies for AFB_1 and the mean classical association constant of $34.5 \times 10^3\ M^{-1}$ with standard free energy change of -6.19Kcal/mol. (Fig. 5) suggest that the antibodies have high affinity for AFB_1. The polyclonal antibodies could therefore be useful *in vivo* and *in vitro* in immunodiagnosis and immunodetection of this mycotoxin in body fluids/tissues of populations at high or low risk of exposure to the toxin. Exposure of humans to aflatoxins had previously been assessed based on the detection of urinary metabolites of the toxin or by the presence of AFB_1 or it's adducts in the peripheral blood and serum [10, 22]. It has been shown that AFB_1-DNA and AFB_1-H_1 adducts undergo rapid turnover after aflatoxin incorporation into the macromolecules before their excretion in urine [8]. Immunodetection of such adducts in urine using the studied antibodies may be more sensitive in giving informations on recent degree of exposure to AFB_1.

It is known that immunization with a carcinogen-protein conjugate may confer some protection against the effects of the specific carcinogen [23]. In this study, 45% of rats dosed with AFB_1 survived the toxic effects within three days of treatment while 70% of those immunized prior to challenge with AFB_1 survived the toxic effects within those days. The high mortality rate observed in the unimmunized rats may be attributed to the degree of acute toxicities of AFB_1. The protective effect of immunization reflected by the decreased mortality of the immunized rats may be due to the presence of AFB_1-specific antibodies produced against the AFB_1-H_1 complex. The antibodies may have intercepted the transport of the toxin to target sites and consequently inhibited it's deleterious effects. The mean serum and liver ALP and GT activities of unimmunized rats dosed with AFB_1 which are higher than those observed for the immunized rats dosed with AFB_1 and the control rats which were not immunized nor dosed with AFB_1 (Fig. 6) shows that AFB_1 toxic effects induced increased synthesis of ALP and GT in the damaged liver as well as leakage of the enzyme into the serum. Limited histological examination on the rat liver following exposure to AFB_1 revealed that the liver from unimmunized rats dosed with AFB_1 show severe bile duct hyperplasia, necrotic and vacuolated hepatocytes and enlarged hyperchromatic hepatocyte nuclei. Marked reduction of these lesions was observed in the liver of rats immunized before challenge with the toxin. This further suggests that immunization of the rats with the complex prior to challenge with AFB_1 do protect them against chronic and acute toxic effects that resulted from exposure to the mycotoxin. Therefore, it may be possible to use AFB_1-H_1 complex and other AFB_1-protein conjugates in animals and may be humans for immunoprophylaxis. Further studies are being directed at verifying whether immunization with the complex will actually inhibit AFB_1 induced hepatocarcinogenic effects and thus its probable use in vaccine development.

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