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Annals of Vaccines and Immunization

Research Article

Anaflatoxin B_1 as the Paradigm of a New Class of Vaccines Based on "Mycotoxoids"

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Abstract

Mycotoxicoses are a group of important diseases caused by human and animal dietary, respiratory, and dermal exposure to mycotoxins, fungal secondary metabolites that exert toxic effects in minimum concentration. Human exposure result mainly from consumption of plant-based foods containing mycotoxins and animal-derived foods, such as milk, meat and eggs, containing mycotoxins residues and metabolites (carry over).

Aflatoxins (AF) are among the most relevant mycotoxins of medical interest due to their high toxic, carcinogenic and immunosuppressive properties for humans and animals. AFs are the most intensively studied mycotoxins in dairy industry as the excretion of AFM_1 in milk is a relevant public health concern.

Other than prevention of mycotoxin exposure, there are currently no known prophylactic measures for mycotoxicoses and no therapeutic treatments are available. Thus, the production of a vaccine able to induce specific neutralizing antibodies would be of great social, scientific and economic interests.

Mycotoxins are non proteinaceous, low molecular weight haptens. Their use as conjugate immunogens should be carefully evaluated owing to the toxic properties of the molecule that might be released. Potential vaccine candidates could be protein conjugates of "mycotoxoids", chemically detoxified forms of mycotoxins still maintaining the ability to induce antibodies reactive with the parent molecule.

The present review gives an overview of overall aspects of immunization against mycotoxins, with emphasis on Anaflatoxin B_1 as a paradigm of "mycotoxoid" which has been used in immunization protocols to prevent AFB_1 carry over as AFM_1 in milk.

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Submitted: 11 May 2015

Accepted: 12 July 2015

Published: 13 July 2015

Keywords

- Mycotoxins
- Aflatoxins
- Mycotoxoid
- Vaccination

ABBREVIATIONS

Ab: Antibody; AF: Aflatoxins; $AnAFB_1$: Anaflatoxin B_1; BSA: Bovine Serum Albumin; CT: Cholera Toxin; F: Fumonisins; KLH: Keyhole Limpet Hemocyanin; OT: Ochratoxins; rLT-B: Recombinant Heat-labile *E. coli* Enterotoxin B Subunit; sIgA: Mucosal Secretory Immunoglobulin A; TG: Thyroglobulin; ZEN: Zearalenone

INTRODUCTION

Fungi cause a broad range of diseases in animals, involving parasitism of organs and tissues as well as allergenic manifestations [1]. Other than poisoning through ingestion of non-edible mushrooms, fungi can produce mycotoxins and organic chemicals that are responsible for various toxic effects [2].

Mycoses are the best-known diseases of fungal etiology and may range from superficial to cutaneous, subcutaneous, or deep-seated infections, leading to a variable stimulation of the host immune response [1,3,4]. The majority of life-threatening fungal infections develop from inhalation of infectious propagules into the airways of susceptible hosts (e.g. *Aspergillus fumigatus, Cryptococcus neoformans, Histoplasma capsulatum*) or penetration through breaches in mucocutaneous barriers (e.g. indwelling vascular access catheters or following abdominal surgeries) by commensal organisms such as *Candida albicans.*

Mycotoxicoses are caused by exposure to mycotoxins, pharmacologically active compounds produced by filamentous fungi contaminating foodstuffs or animal feeds [5]. Mycotoxins are secondary metabolites not critical to fungal physiology, that are extremely toxic in minimum concentrations to

vertebrates upon ingestion, inhalation or skin contact [5,6]. About 400 mycotoxins are currently recognized, subdivided in families of chemically related molecules with similar biological and structural properties. Of these, approximately a dozen groups regularly receive attention as threats to animal health. Examples of mycotoxins of greatest public interest and agro-economic significance include aflatoxins (AF), ochratoxins (OT), trichothecenes (T), zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids [5]. Mycotoxins have been related to acute and chronic diseases, with biological effects that vary mainly according to the diversity in their chemical structure, but also with regard to biological, nutritional and environmental factors [2]. The pathophysiology of mycotoxicoses is the consequence of interactions of mycotoxins with functional molecules and organelles in the animal cell, which may result in carcinogenicity, genotoxicity, inhibition of protein synthesis, immunosuppression, dermal irritation, and other metabolic perturbations [6]. In sensitive animal species, mycotoxins may elicit complicated and overlapping toxic effects. In Table 1 are listed the main features of the mycotoxins of greatest public health hazard and agro-economic significance [7,8].

Mycotoxicoses are not contagious, nor is there significant stimulation of the immune system. Treatment with drugs or antibiotics has little or no effect on the course of the disease [9].

To date no human or animal vaccine is extensively available for combating either mycoses or mycotoxicoses. The design of effective vaccination formulations to prevent or mitigate fungal infections has probably lagged behind because potentially pathogenic fungal species seldom cause the disease in mammalian hosts, which are highly resistant owing to a combination of a complex immune system and endothermy [10]. In fact, among hundreds of thousands of fungal species which have been formally described, only several hundreds can cause human disease [11,12]. Possibly excluding few species that can cause infections in the healthy population (anthropophilic dermatophytes), the majority of fungi are opportunistic pathogens (such as *Candida* spp. and *Aspergillus* spp.) that usually can be cause of disease only in immunocompromised hosts [10]. Mycoses, however, have increased substantially over the past 40 years and fungi became important human pathogens in hosts with predisposing conditions (e.g. impaired immunity as a consequence of medical interventions or HIV infection) [10]. In sub-Saharan Africa, for example, cryptococcal meningitis exceeded well-known infections such as tuberculosis as a cause of HIV-associated deaths [1]. In immunosuppressed patients, invasive fungal infections carry a high mortality rate even in the presence of antifungal therapy, which is limited by toxicity, emergence of resistance and high cost [13]. In addition, the range and diversity of fungi that cause disease have broadened. A growing body of work is thus focusing in developing vaccines and/or immunotherapy with efficacy against broad fungal classes as a powerful tool in combating mycoses, either for the active treatment, as an adjuvant, or in the prevention of specific fungal diseases [10].

In contrast to mycoses, mycotoxicoses do not need the involvement of the toxin producing fungus and are considered as abiotic hazards, although with biotic origin [9]. In this sense, mycotoxicoses have been considered examples of poisoning by natural means, and protective strategies have essentially focused on exposure prevention [5].

Human and animal exposure occurs mainly from ingestion of the mycotoxins in plant-based food. Metabolism of ingested mycotoxins could result in accumulation in different organs or tissues; mycotoxins can thus enter into the food chain through animal meat, milk, or eggs (carry over) [9].

Because toxigenic fungi contaminate several kinds of crops for human and animal consumption, mycotoxins may be present in all kinds of raw agricultural materials, commodities and beverages. The Food and Agriculture Organization (FAO) estimated that 25% of the world's food crops are significantly contaminated with mycotoxins [14].

At the moment, the best strategies for mycotoxicoses prevention include good agricultural practice to reduce mycotoxins production on crop and control programs of food and feed commodities to ensure that mycotoxin levels stand below legally fixed threshold limits [5,15,16]. These strategies may limit the problem of contamination of commodities with some groups of mycotoxins with high costs and variable effectiveness.

Except for supportive therapy (e.g., diet, hydration), there are almost no treatments for mycotoxin exposure and antidotes for mycotoxins are generally not available, although in individual exposed to AFs some encouraging results have been obtained with some protective agents such as chlorophyllin, green tea polyphenols and dithiolethiones (oltipraz) [17].

Vaccination against specific mycotoxins could be proposed to prevent mycotoxicosis in livestock and contamination by mycotoxins in important foods of animal origin with an entirely

Table 1: Features of most common mycotoxins.

Mycotoxins	Toxigenic fungi	Occurrence	Biological effect
Aflatoxins	*Aspergillus flavus*, *A. parasiticus*	Corn, peanuts, rice, wheat, cottonseed, tree nuts, dairy products	Carcinogenic, mutagenic, immunotoxic, hepatotoxic, teratogenic, immunosuppressive
Ochratoxins	*A. ochraceus*, *A. niger*, *Penicillium verrucosum*, *P. carbonarius*	Cereals, coffee, cacao, chocolate, spices, dried fruit, red wine	Nephrotoxic, carcinogenic, teratogenic, immunotoxic, nephrotoxic
Fumonisins	*Fusarium verticillioides*, *F. proliferatum*	Maize (corn)	Carcinogenic
Zearalenone	*Fusarium* spp., *F. graminearum,*	Maize, wheat	Estrogenic
Trichothecenes	*Fusarium* spp., *Trichoderma* spp., *Trichothecium* spp., *Myrothecium* spp., *Stachybotrys* spp.	Flour, rice, barley, oats, corn	Haemorrhagic, dermotoxic

innovative strategy based on the production of antibodies (Abs) that could specifically block initial absorption or bioactivation of mycotoxins, their toxicity and/or secretion in animal products (such as milk) by immuno-interception (neutralization).

Strategies for immunization against mycotoxins

The production of vaccines for protection against mycotoxicoses presents some theoretical and technological challenges, principally related to the wide range of structures, chemical properties, and toxicity associated with mycotoxins. Mycotoxins are small molecular weight, usually nonproteinaceous molecules, which are not ordinarily immunogenic (haptens), but can elicit an immune response when attached to a large carrier molecule such as a protein. Methods for conjugation of mycotoxins to protein or polypeptide carrier and optimization of conditions for animal immunization have been extensively studied, with the purpose of producing monoclonal (mAbs) or polyclonal Abs with different specificities to be used in immunoassay for screening of mycotoxins in products destined for animal and human consumption. Coupling proteins used in these studies included bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), thyroglobulin (TG), polylysine, among others [18].

Because of the diversity of chemical structures and physicochemical properties of mycotoxins, approaches for their coupling procedures vary considerably. Some mycotoxins such as OA contain an active carboxyl group, which can be directly conjugated to a protein carrier for Abs production [19]. Others, such as T-2, AFB_1 and most other mycotoxins, require first to be derivatized by introduction of a reactive group into the mycotoxin molecule before it can be coupled to a protein [20]. In the past decades, many efforts have been made for developing mycotoxin derivatives that can be bound to proteins while retaining enough of the original structure so that Abs produced will recognize the native toxin. For this purpose, the immunizing hapten should represent a near perfect mimic of the target molecule in structure, electronic and hydrophobic properties and should have an attachment arm of 3-6 carbons length with an active group, which can easily be used for conjugation with the carrier protein [21]. For example, groups having acarboxyl functionality can be activated and covalently coupled to amine groups in proteins to form stable amide linkages. Several amide-bond formation reactions have been employed including those that make use of the N-hydroxysuccinimide activated esters, water-soluble carbodiimides, and mixed anhydrides [19]. Through these methods, Abs against almost all the important mycotoxins have been made available, demonstrating that conjugation to proteins may be an effective tool for immunization [22]. The application of this strategy for human and animal vaccination, however, should be carefully evaluated for the toxic properties of the molecules that might be released *in vivo*. For example, conjugation of toxins such as T-2 to protein carriers has been shown to result in unstable complexes with potential release of the free toxin in its active form [23].

In analogy with toxoid vaccines, which may confer a state of protection against the pathological effects of bacterial toxins, a reasonable approach to the development of vaccines against mycotoxin may be based on conjugated "mycotoxoids", defined as modified form of mycotoxins, devoid of toxicity although maintaining antigenicity.

Bacterial toxins are proteins, which may be converted to toxoids inducing alterations in particular amino acids with minor molecular conformational changes, e.g. through formaldehyde treatment [24].

Given the nonproteinaceous nature of mycotoxins, the approach for conversion to mycotoxoids should rely on chemical derivatization. The introduction of specific groups in strategic positions of the related parent mycotoxin may lead to formation of molecules with different physicochemical characteristics, but still able to induce Abs cross-reacting to the native toxin. The rationale for mycotoxin vaccination would thus be based on generating Abs against the mycotoxoid with an enhanced ability to bind native mycotoxin compared with cellular targets, neutralizing the toxin and preventing disease development in the event of exposure.

A potential application of this strategy has been demonstrated in the case of mycotoxins belonging to the AF group.

Mycotoxoids for vaccination against mycotoxins: the paradigm of aflatoxins

AFs are a group of bis-furocoumarin metabolites produced mainly by strains of *Aspergillus flavus* and *A. parasiticus*. Over 20 AFs and derivatives have been isolated, but the major natural occurring AFs of fungal origin are B_1, B_2, G_1, and G_2, with the B_1 (AFB_1) being the most important compound with respect to both prevalence and toxicity for man and animals (Figure 1) [25, 26].

Even if cases of acute intoxication may also occur, chronic toxicity of AFs is the major public health concern [27,28]. It has been estimated that more than 4.5 billion people are chronically exposed to AFs as natural contaminants in food [28]. Chronic consumption of AFs has been identified as one of the major risk factors for the development of hepatocellular carcinoma and AFs are classified as Group 1 carcinogens by International Agency for Research on Cancer [27]. Evidence suggests a relation between chronic AFs exposure and malnutrition, impaired growth, immunosuppression, and, consequently, susceptibility to infectious diseases. In consideration of these findings, it has been reviewed that there is a reasonable probability that several top World Health Organization (WHO) risk factors are modulated by AFs [28].

Primary aflatoxicoses occurs after human and animal exposure to AFs, mainly through ingestion of contaminated food and feeds [29]. However, as a result of the carry over process, when AF-contaminated feeds are fed to livestock, the AFs or their metabolites pass into the meat, milk, and eggs. Human consumption of these products may determine chronic exposition to sublethal doses of AFs, eventually leading to secondary aflatoxicosis. In particular, part of ingested AFB_1 is biotransformed by hepatic microsomal cytochrome P_{450} into various metabolites, including the 4-hydroxy derivative aflatoxin M_1 (AFM_1) which is then excreted into the milk of lactating mammals, including dairy animals [30]. This phenomenon is of great concern for the high toxicity of AFM_1 and because of the importance of milk for human nutrition, especially for children

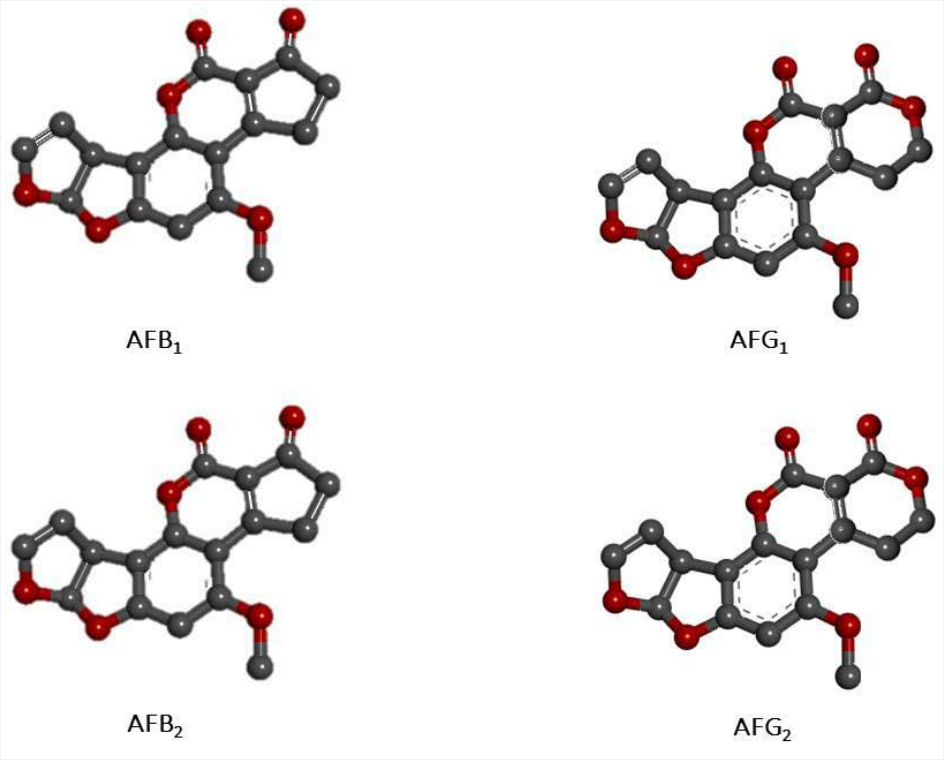

Figure 1 Structures of the most important naturally occurring AFs. Carbon, gray; oxygen, red.

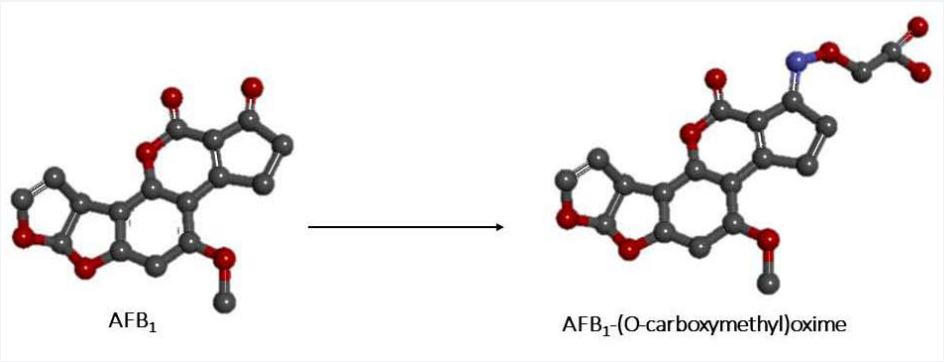

Figure 2 Preparation of AFB1-(O-carboxymethyl)oxime. Carbon, gray; oxygen, red; nitrogen, blue.

who are potentially more susceptible to AFs detrimental health effects.

To prevent primary aflatoxicosis, rigorous limits for AFB_1 in food have been set. Limits for AFB_1 in the feed of dairy animals have also been established in order to ensure the respect of the maximum limit of AFM_1 in the milk destined to human use [31]. Such regulations, however, protect a population from exposure to highly contaminated lots, but do not remove AFM_1 from the international food chain.

For the rationale management of the hazard associated with AFM_1 contamination of milk, a new preventive approach has recently been proposed which relies on vaccination of dairy cows to exploit specifically induced Abs to hinder AFB_1 carry over as AFM_1 in milk [32,33].

AFs are small molecular weight, toxic and mutagenic haptens, which may raise an immune reaction only when attached to large carrier molecules, such as proteins. As described for other mycotoxins, in the last decades, many efforts have been directed in the synthesis of different AF derivatives and protein conjugates for immunization and production of Abs for immunochemical assay for toxin determination [34].

Only a few studies were carried out in order to find whether immunization with AF derivatives conjugated to carrier proteins might be used prophylactically against aflatoxicosis.

In early promising experiments, reduction of acute toxic effects of AFB_1 was achieved by immunizing rodents with AFB_1 derivatives conjugated to BSA. After immunization with small amounts of AFB_1-BSA and challenge with a single dose of AFB_1

by intraperitoneal injection, rabbits showed higher survival rates, near normal serum isocitric dehydrogenase activity, no abnormality in livers [35]. Similar results were obtained by injection of water in oil emulsion of AFB_1 derivatives conjugated to either BSA or histone H1 into male Wistar albino rats [36,37]. In an additional experiment, a reduction of the covalent binding of AFB_1 to liver DNA of rabbits immunized with AFB_1-BSA was observed [38]. The Covalent Binding Index, CBI (μmol AFB_1/mol DNA)/(mmol AFB_1/kg body weight) obtained 6 h after oral administration of [^{3}H]AFB_1 to immunized rabbits was found to be more than 3 times lower than in control (non immunized) rabbits. As long as covalent binding of AFB_1 to liver DNA has been shown to reflect susceptibility for liver tumor formation in various animal species, these results are encouraging evidence for the potential of active immunization against genotoxic effect of AFs [39]. Quite interestingly, the AFB_1 derivative was shown to be nontoxic to chicken embryos as compared to the parent toxin AFB_1 [40]. So it seems possible its use as protein conjugate in view of a potential vaccine formulation.

Confirmation of the potential of specific Abs in the protection against the genotoxic effects of AFB_1 was obtained *in vitro* with *Salmonella typhimurium* (Ames Test) and *Escherichia coli* K12 bacteria lysogenic for the λ phage in presence of different metabolic activation systems [41,42]. In these assays, rabbit anti-AFB_1 antisera raised against different AFB_1-BSA conjugates demonstrated substantial inhibition of AFB_1-induced mutagenicity and lysogenesis induced in *S. typhimurium* and *E. coli* K12, respectively.

Based on evidence that a carcinogen-specific mucosal secretory IgA (sIgA) response could block carcinogen absorption across the mucosa [43,44], an attempt to generate an sIgA immune response potentially protective against AFB_1 was conducted in chickens [45]. In this study, animals were immunized perorally, intrarectally, or intraperitoneally, with one of four vaccine preparations: 1) AFB_1-BSA conjugate alone, 2) AFB_1-BSA linked to the B subunit of the recombinant heat-labile enterotoxin of *E. coli* (rLT-B), 3) AFB_1-BSA mixed with rLT-B, or 4) AFB_1-BSA mixed with cholera toxin (CT). All three routes of immunization elicited significant serum IgG responses, with highest responses induced following intraperitoneal administration. The serum IgG immune response to AFB_1-BSA was enhanced by co-administration of rLT-B but not by covalent coupling to rLT-B or coadministration with CT. In all treatment groups, sIgA anti-CT and anti-rLT-B Abs were detected in faecal supernatants, but no sIgA anti-AFB_1 Abs could be detected.

In another attempt to generate an sIgA immune response to AFB_1, rabbits were co-immunized through chronically isolated ileal loops with an AFB_1 derivative coupled to porcine thyroglobulin (TG) and CT, a potent mucosal adjuvant [46]. This protocol resulted in a negligible sIgA anti-AFB_1 response [47, 48]. On the contrary, strong mucosal Ab responses to CT and TG were generated by this immunization protocol, suggesting that the observed unresponsiveness was specific to AFB_1 and ruling out AFB_1-mediated immunosuppression.

Mitogen stimulation of cells isolated from mesenteric lymph nodes, Peyer's patch and spleens of unimmunized animals revealed the presence of AFB_1-specific and TG-specific Ab secreting cells (ASC) at comparable levels in both mucosal and systemic lymphoid tissues. Although this method did not allow a quantitative estimate of the precursor frequency of AFB_1-specific B cells in these tissues, it demonstrated that AFB_1-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 antigens that are as much as 10- to 1000-fold stronger than those generated by antigen alone [49,50]. Altogether, these results demonstrate that unresponsiveness to AFB_1 in rabbits is hapten-specific, restricted to the mucosal immune system, and refractory to the adjuvancy of CT. This is consistent with the observation that an established mucosal tolerance cannot be broken by coadministration with LT of *E. coli*, which is structurally and functionally similar to CT, suggesting that bacterial enterotoxins may not be effective mucosal adjuvants for antigens to which tolerance has already been established [51]. This phenomenon may thus be a result of prior dietary exposure to AFB_1. All the above described investigations have focused primarily on immunizing against AFB_1 in the attempt to protect animals from primary aflatoxicosis.

The first attempt to develop an immunoprophylactic approach to protect human consumers from secondary aflatoxicosis has been conducted with a vaccine administered systemically to cows to minimize the amount of carry-over of AFM_1 into the milk [32]. The experimental approach was essentially based on the use of a conjugate vaccine, consisting of AFB_1 chemically modified as AFB_1-1(O-carboxymethyl)oxime, conjugated to KLH (Figure 2). In contrast to AFB_1, AFB_1-1(O-carboxymethyl) oxime proved to be non-toxic *in vitro* to human hepatocarcinoma cells and non mutagenic to *S. typhimurium* strains, while retaining the original AFB_1 antigenic properties. Considering these results and the lack of toxicity in other biological systems [40], AFB_1-1(O-carboxymethyl)oxime was designated as Anaflatoxin B_1 ($AnAFB_1$). When $AnAFB_1$-KLH conjugates were administered to cows as systemic immunogens with complete and incomplete Freund's adjuvant, they elicited specific anti-AFB_1 long lasting Igs, mostly pertaining to IgGs, which also cross-reacted with AFG_1, AFB_2, and AFG_2. Interestingly, the authors reported that a single dose administration of the vaccine in Freund's complete adjuvant (first inoculum) did not induce delayed hypersensitivity to *Mycobacterium tuberculosis* in any of the vaccinated cows, as evidenced by negative intradermal tuberculin test. This finding should exclude any interference with the primary diagnostic test for bovine tuberculosis. According to Ab titer specific for AFB_1, it was possible to recognize, among the 6 vaccinated cows, 3 high responder and 3 low responder animals. The efficacy of specific Abs in preventing or reducing AFB_1 transfer into milk was evaluated by monitoring AFM_1 concentrations in milk of lactating cows. An intermittent exposure regimen with two intoxication periods was designed to evaluate efficacy of anti-AFB_1 Abs over time and in two different lactation (mid and late) stages. In these experiments, the titer of Abs specific to AFB_1 in vaccinated cows correlated well with the prevention of carry-over in the milk, following exposure of the cows to feed contaminated with AFB_1. In particular, high responder cows produced an average milk AFM_1 concentration at the steady-state 46% and 37% lower of that observed in control cows during mid and late lactation stage,

respectively. As long as specific anti-AFB_1 Abs elicited in the high responder cows appeared to reduce the excretion of AFM_1 in the milk following intoxication either in the mid or late lactation stages, it was possible to conclude that vaccination may confer protection over the whole production cycle, before the drying off period.

In an attempt to obtain a more potent Ab response and then to increase prevention of AFB_1 carry-over as AFM_1 from feed to milk in vaccinated cows, the effect of conjugation of $AnAFB_1$ with other protein carriers, the effect of administration with various immunological adjuvants, and the effect of animal age were subsequently studied [33]. The results obtained suggest that pre-calving administration can increase the effectiveness of vaccination, resulting in 100% high responder animals. Anti-AFB_1 Ab titers of vaccinated heifers decreased during pregnancy and after calving but, after one booster dose at the beginning of the milk production cycle, titers returned to levels comparable to the end of the immunization schedule. Monitoring of AFM_1 concentrations in milk, as compared to non vaccinated cows, demonstrated the effectiveness of anti-AFB_1 Abs in reducing from 3.40% to 0.78% the carry-over of AFB_1 as AFM_1 in vaccinated heifers, resulting in a 74% reduction of AFM_1 contamination.

Reduction of AFM_1 in milk obtained in vaccinated cows was notably high, as compared to reductions that can be obtained with alternative strategies which have been envisaged to face the risk of exposition to AFM_1, such as detoxification methods or enterosorption with sequestering agents. For example, addition of AFs sequestering agents to animal diets could reduce AFM_1 transfer in the milk of no more than 50% [52, 53]. Moreover, considering that specific Abs and sequestering agents reduce AFs carry-over by acting at different stages of AFs toxicokinetic pathway, it is possible to speculate that their effects may be cumulative. Vaccination of lactating animals with $AnAFB_1$-KLH may then represent a valid tool for the complete prevention of AF contamination of milk and dairy products.

DISCUSSION AND CONCLUSION

Although the way to provide a suitable vaccination against AFs is still in its early steps, these studies demonstrate the feasibility of controlling AFM_1 carry over in dairy cows by a strategy aimed at neutralizing AF through a proper stimulation of the animal immune system.

These findings constitute the reliable basis for further investigation on the protective effects of vaccination against aflatoxicoses in other animal species and, prospectively, in man. Moreover, $AnAFB_1$-KLH may represent a prototype of a new class of conjugated mycotoxoid vaccines, based on molecules produced by chemical modification of mycotoxins in order to obtain antigenic mimics of the parent molecule devoid of toxicity.

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Cite this article

Giovati L, Ciociola T, Conti S, Magliani W, Sperindè M, et al. (2015) Anaflatoxin B1 as the Paradigm of a New Class of Vaccines Based on "Mycotoxoids". Ann Vaccines Immunization 2(1): 1010.