An immunogen synthesis strategy for the development of specific anti-deoxynivalenol monoclonal antibodies

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An immunogen synthesis strategy was designed to develop anti-deoxynivalenol (DON) monoclonal antibodies with low cross-reactivity against structurally similar trichothecenes. A total of eight different DON immunogens were synthesised, differing in the type and position of the linker on the DON molecule. After immunisation, antisera from mice immunised with different DON immunogens were checked for the presence of relevant antibodies. Then, both homologous and heterologous enzyme-linked immunosorbent assays (ELISAs) were performed for hybridoma screening. Finally, three monoclonal antibodies against DON and its analogues were generated. In addition, monoclonal antibody 13H1 could recognise DON and its analogues in the order of HT-2 toxin > 15-acetyldeoxynivalenol (15-ADON) > DON, with IC50 ranging from 1.14 to 2.13 µg ml−1. Another monoclonal antibody 10H10 manifested relatively close sensitivities to DON, 3-acetyldeoxynivalenol (3-ADON) and 15-ADON, with IC50 values of 22, 15 and 34 ng ml−1, respectively. Using an indirect ELISA format decreases the 10H10 sensitivity to 15-ADON with 92%. A third monoclonal antibody 2A9 showed to be very specific and sensitive to 3-ADON, with IC50 of 0.38 ng ml−1. Using both 2A9 and 10H10 monoclonal antibodies allows determining sole DON contamination.

Keywords: deoxynivalenol; monoclonal antibodies; immunogens; ELISA; cross-reactivity

Introduction

Trichothecene mycotoxins are a group of naturally occurring secondary metabolites produced by Fusarium species, in particular F. graminearum and F. culmorum. Among the 150 related trichothecenes, deoxynivalenol (DON) (Figure 1) is of special importance as it is formed in the field prior to harvest and because its occurrence cannot be completely avoided by plant production-minimising strategies due to the high impact of weather conditions. Especially wheat, triticale and maize are vulnerable for Fusarium infection and subsequent DON production (Maragos et al. 2006; Döll & Dänicke 2011).

DON can cause disease in several animal species, especially in swine, and causes symptoms including reduced feed consumption, abdominal distress, increased salivation, malaise, diarrhoea, anorexia, leukopenia, haemorrhage, shock and death in extremely high DON doses (Maragos & McCormick 2000; Pestka 2007). Besides these effects, DON is also generally considered to be a potent inhibitor of protein and DNA synthesis and is known to be immunosuppressive (Krska et al. 2001; Danicke et al. 2006). Highly dividing cells, such as intestinal epithelial cells known to ensure a proper barrier function, are especially sensitive to trichothecenes. Exposure to DON can lead to a decrease in absorption of nutrients and a decrease in cell proliferation and consequently cell differentiation (Pinton et al. 2012).

Next to DON two acetylated derivatives are known to be coproduced, namely 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). JECFA stated that the acetylated DON levels were less than 10% of the total DON found in cereal grains, whereas De Boevre et al. (2012) found higher 3-ADON and 15-ADON co-contamination for several food and feed samples. In 2010 JECFA considered the toxicity of the acetylated derivatives equal to DON, but a recent study suggested that higher toxicity of 15-ADON should be taken into account. Another DON masked form detected in cereals and beers, namely DON-3-gluco side (DON-3-G), also shows a non-negligible contribution to the overall DON contamination (Berthiller et al. 2009; JECFA 2011; De Boevre et al. 2012; Pinton et al. 2012).
Because of the economic importance of this toxin and from a food safety perspective, a variety of analytical techniques have been developed for the detection of DON and related trichothecene mycotoxins in food and feed. Most commonly used techniques include TLC, GC, HPLC and immunological approaches such as radioimmunoassay and ELISA. Recently, ELISA and ELISA-based procedures have gained acceptance as they offer the advantage of specificity, sensitivity, simplicity and rapidity, which are of importance for routine testing of mycotoxins (Maragos & McCormick 2000; Maragos et al. 2006, 2012). The specificity and sensitivity are dependent on the antibody used in the ELISA assay. Several antibodies for DON have been reported and all show high cross-reactivity against the acetylated derivatives 3-ADON and 15-ADON, which reduces their specific character. Sensitivity values of antibodies, measured by the IC$_{50}$ value, range from approximately 20 to 3 ng ml$^{-1}$ (Casale et al. 1988; Mills et al. 1990; Usleber et al. 1991; Sinha et al. 1995; Maragos & McCormick 2000).

The objective of this study is to improve the specificity of the ELISA assay by developing highly specific monoclonal anti-DON antibodies. The high affinity of the monoclonal antibody to 3-ADON or 15-ADON likely derives from the chemistry used to prepare the immunogen. The ester linkage of 3-ADON or 15-ADON may resemble the linkage of the DON-carrier protein immunogen. Therefore, it was decided to use different procedures to make linkers with varying length and chemical structure between DON and the carrier protein (Figure 2).

**Materials and methods**

**Reagents**

DON, 3-ADON and 15-ADON standards were obtained from Fermentek (Jerusalem, Israel). HT-2 toxin was purchased from Sigma-Aldrich (Bornem, Belgium).

Colorburst$^TM$ blue 3,3’,5,5’-tetramethylbenzidine (TMB) substrate solution containing hydrogen peroxide was supplied by Alerchek (Springvale, ME, USA). Rabbit anti-mouse immunoglobulins (anti-mouse IgG secondary antibody; protein concentration of 2.5 g l$^{-1}$) were purchased from DakoCytomation (Glostrup, Denmark). N, N’-carbonyldiimidazole, cyanuric chloride (CC), N,N’-diisopropylethylamine, glutaric anhydride, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), 1-butane boronic acid, sodium tetraborate, copper (II) sulfate, sodium ascorbate, 4,7-diphenyl-1,10-bathophenanthroline disulfonic acid disodium salt, arginine, glycine, aspartic acid, D-phenylalanine, lysine, trifluoroacetic acid (TFA), triisopropylsilane (TIS), methyl tertiary butyl ether (MTBE), sodium tetraborate, horseradish peroxidase (HRP), rabbit anti-mouse IgG secondary antibody labelled with horseradish peroxidase (Sec Ab-HRP), PBS (0.01 M, pH 7.4) tablet, carbonate-buffered saline (CBS, 0.05 M, pH 9.6) capsule, complete and incomplete Freund’s adjuvants, Tween 20 and skim milk powder were obtained from Sigma-Aldrich (Bornem, Belgium). O-propargyl-hydroxylamine hydrochloride was purchased from Focus Synthesis (San Diego, CA, USA). Imidazole-1-sulfonylazide hydrochloride was kindly provided by the Laboratory of Medicinal Chemistry (Ghent, Belgium). The Oasis HLB cartridges were obtained from Waters (Zelik, Belgium). Chlorotrityl chloride resin was purchased from Merck (Darmstadt, Germany). Deionised water was purified by a Millipore Milli-Q system (Brussels, Belgium). Other chemicals and solvents were of analytical grade.

**Figure 2.** Three series of synthesised DON immunogens.
Nunc-Immuno™ F96-well microplates and Nunclon™ cell culture plates were from Nalge Nunc International (Roskilde, Denmark). Protein concentrators (9K MWCO, 20 ml) were purchased from Thermo Scientific (Rockford, USA).

**Preparation of DON immunogens**

DON is a hapten due to its small size and therefore cannot elicit an immune response. Consequently, for the synthesis of immunogens, DON was coupled to a protein via a linker to increase the molecular weight. In search for specific anti-DON antibodies, different synthetic strategies were followed. Firstly, DON was coupled to a carrier protein via the C3 and/or C15 position using a linker with a carboxyl function (Series I, Figure 2). To minimise the chance of cross-reacting monoclonal antibodies, a second DON series of immunogens was generated by coupling the carrier protein via a linker on C3 and/or C15 without a carboxyl function (Series II, Figure 2). To reduce even further the possibility of cross-reacting monoclonal antibodies the linkage to the carrier protein was introduced through reaction with the C8 carbonyl function of DON, delivering a last series of immunogens (Series III, Figure 2). The used carrier proteins were bovine serum albumin (BSA) for the immunogen and ovalbumin (OVA) or horseradish peroxidase (HRP) for the coating/competitive antigen in indirect or direct enzyme-linked immunosorbent assay (ELISA), respectively. The synthetic pathways underneath are described for BSA. The basic chemical structure of the three different series of immunogens is illustrated in Figure 2.

The first immunogen of the series I is DON-3,15-hemiglutaryl (HG)-BSA (Figure 3A) and was formed by the reaction of DON with glutaric anhydride in dry pyridine. Concisely, to a solution of 5 mg of DON in 500 µl of dry pyridine, 100 mg of glutaric anhydride was added and the solution was heated at 100°C for 8 h. After evaporation, the residue was dissolved in 7.5 ml of chloroform and washed three times with 5 ml of 0.1 M HCl. A purification step was performed using Oasis HLB® cartridges by a method derived from De Smet et al. (2010). Briefly, 5 µg of the crude DON-HG conjugate was dissolved in 1 ml of methanol–water (10:90, v/v) pH 2.3 and loaded onto a preconditioned column. After loading, the sorbent was dried for 15 s by applying vacuum. DON-HG was eluted by washing the column with 1 ml of methanol–water (55:45, v/v) followed by 1 ml of methanol. The sorbent was dried for 15 s by applying a vacuum. After purity determination by time-of-flight (TOF) MS, an aliquot of 1 mg of the synthesised DON-HG (1.92 µmol) was dissolved in 0.5 ml PBS using ultrasonication and transferred to 30 mg BSA (0.45 µmol) in 1 ml of PBS. Then 20 mg of EDC (0.10 µmol) was added to the above mixture and incubated for 5 h at RT with stirring (Mills et al. 1990; Usleber et al. 1991; Kohno et al. 2003; De Smet et al. 2010).

To obtain DON with one HG linker, namely DON-3-HG-BSA (Figure 3B), prior protection of the 7-OH and 15-OH groups as a cyclic boronate ester allowed one to use similar reaction conditions as for DON-3,15-HG-BSA (Casale et al. 1988). Additionally in the first series of immunogens, a common DON-HG carbamate conjugate (Figure 3C and 3D) was synthesised by the N,N’-carbodimidazole (CDI) coupling reaction, adopted from the published literature (Xiao et al. 1995; Maragos & McCormick 2000).

Within-series II, DON-CC-BSA (Figure 3E and 3F) was synthesised by first dissolving 1 mg of DON (3.374 × 10^-3 mmol) in 480 µl of cold acetonitrile. A solution of 620 µg of cyanuric chloride (3.362 × 10^-3 mmol) in 1.24 ml of acetonitrile was prepared and cooled until –20°C. The DON solution was added over 1 h to the vigorously stirred solution of cyanuric chloride. A solution of 870 µg N,N’-disopropylethylamine in 260 µl of cold acetone was added to the solution of DON and cyanuric chloride and mixed for 5 h at 55–60°C followed by mixing for 16 h at RT. For coupling to BSA, a solution of 1830 µg of BSA (0.0270 µmol) in 1090 µl of 0.1 M sodium tetraborate (pH 9.2) was prepared. The solution was cooled to 4°C and 3.5 mg of DON, a 10-fold molar excess with respect to the available amino groups was added and the resulting mixture was stirred for 1 h at 4°C (Abuchowski et al. 1977; Abuknesha & Griffith 2005).

In an effort to reduce the cross-reactivity against 3-ADON and 15-ADON, we decided to use the C8 carbonyl function of DON to attach an appropriate linker via an oxime moiety (Series III). Hereto a carboxymethoxime (CMO) strategy was applied (Figure 3G) (Burkin et al. 2000). To investigate the effect of the length of the linker between DON and the protein and with the aim of lowering the immune response against the linker itself, we also introduced a carboxypropylimine (CPI) linker at the C8 position of DON (Figure 3H). For this purpose, 0.5 mg of DON (1.7 µmol) was reacted with 750 µg of CMO (7 µmol) in 500 µl of dry pyridine for the DON-CMO synthesis and with 1.6 mg of γ-aminobutyric acid (15.5 µmol) in 1 ml dimethylformamide (DMF) for the DON-CPI synthesis. After overnight reaction at RT, the reaction mixture was concentrated and the residue redissolved in 200 µl of water/DMSO (1:1.5, v/v). Next, 2 mg of sulfo-NHS and 4 mg of EDC were added together with 1.775 mg of BSA and the volume was adjusted to 1 ml by further dilution with water. The reactions were performed for 2 h at RT followed by washing and purification as described for previous immunogen synthesis.

Finally, click chemistry was adopted for linking C8 of DON without a carboxyl function. Following a modified procedure of Ikuina et al. (2003), DON was condensed to
Figure 3. Overview of the deoxynivalenol immunogens (only the amino group of the protein is presented): A, DON-3,15-HG protein; B, DON-3-HG protein; C, DON-3 protein; D, DON-15 protein; E, DON-3-CC protein; F, DON-15-CC protein; G, DON-CMO protein; H, DON-CPI protein; I, DON-oxime; J, DON-azido protein; K, DON-cyclic peptide protein.
O-propargy-hydroxylamine hydrochloride (Figure 3I). Briefly, 2 mg of DON (6.76 µmol) was reacted with 1.81 mg of O-propargy-hydroxylamine hydrochloride (25.52 µmol) in the presence of THF/acetic acid (1:1, v/v) at 40°C. After 2 h, an additional 170 µg of O-propargy-hydroxylamine hydrochloride was added and the mixture was stirred for another 4.5 h to obtain DON-oxime. For the synthesis of azido-BSA, 10.686 mg of BSA (0.16 µmol) was dissolved in water together with 4 mg of potassium carbonate and 0.5 mg of copper (II) sulfate. Then, 1 µg of imidazole-1-sulfonylazide hydrochloride (4.79 µmol) was added and the reaction was agitated overnight. For the click reaction, 0.5 mg of DON-oxime (1.43 µmol) was transferred to an aqueous solution of azido-BSA (1.42 mg) containing 25 µg copper (II) sulfate (10 mM), 20 µg sodium ascorbate (10 mM) and 54 µg 4,7-diphenyl-1,10-bathophenanthroline disulfonic acid disodium salt (10 mM). The reaction went on for 14 h at RT (Figure 3J) (Ikuina et al. 2003; Van Dongen et al. 2009; Horak et al. 2010).

The click chemistry immunogen product was further expanded by the introduction of a N-azido cyclic peptide (630.3 g mol⁻¹) (Figure 3K). The cyclic peptide increases the distance between DON and BSA and renders the DON molecule more available for the immune system. Through solid-phase peptide synthesis using 2-chlorotrityl chloride resin and a Fmoc/Bu protection scheme, the amino acids glycine, aspartic acid, D-phenylalanine, lysine and arginine were successively coupled to each other. After mild acid cleavage of the peptide from the resin, cyclisation of the peptide was performed followed by treatment with TFA–TIS–H₂O (95:2.5:2.5, v/v/v) to remove the protecting groups in solution. The peptide was then precipitated in MTBE–hexane (1:1, v/v) and redissolved in methanol for a diazo transfer. For the click chemistry, 1 mg of DON-oxime (2.84 µmol) was reacted with 1.78 mg of cyclic peptide (2.84 µmol) under the previously mentioned conditions. Under CDI reaction conditions, the obtained DON-cyclic peptide (2.84 µmol) was coupled to 3.9 mg of BSA in water–DMSO (1/5, v/v) for 3 h at RT (Dai et al. 2000; Dijkstra et al. 2007).

All DON immunogens were dialysed against 4 L PBS to remove low molecular weight substances and concentrated using Pierce Concentrator columns 20 ml/9K MWCO.

**Characterisation of immunogens**

The successful synthesis of the DON-3,15-HG-linker was confirmed by mass spectrometry LCT Premier XETM TOF (Waters, Milford, MA, USA) equipped with a standard electrospray ionisation and modular LockSpray TM interface in the positive and negative electrospray ionisation (ESI⁺/⁻) mode. The purified DON-3,15-HG mixture was infused in acetonitrile–water (1:1, v/v) at 10 µl min⁻¹. The purity of the final product was assessed by HPLC and photo diode array (PDA) detection (190–400 nm) using a Phenomenex Luna 2.5 mm C₁₈ (2-HST) column. A mobile phase consisting of eluents A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) was used at a flow rate of 0.4 ml min⁻¹. A linear gradient of 10–100% solvent B was applied over 9 min. Other mass measurements of DON-linker syntheses were performed by direct injection of the reaction product into the Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) using the ESI⁺/⁻ mode. Masslynx version 4.1 was used for data acquisition.

After coupling to the protein, the immunogen concentration was determined based on the amount of protein coupled by the use of the NanoDrop 2000c (Thermo Scientific, Rockford, USA) and the immunogen was characterised by the use of indirect competitive ELISA. All incubations except for the first coating step were carried out at 37°C. After each incubation, the plates were washed three times (300 µl/well) with PBST (PBS containing 0.05% Tween 20). High-binding Nunc-ImmunoTM F96-well microplates were coated with 10 µg ml⁻¹ of the synthesised conjugate in CBS (100 µl/well). After incubation at 4°C overnight, the plates were blocked with 2% skimmed milk in PBS (300 µl/well) for 30 min. Standard solutions of DON and PBS control were added (50 µl/well), followed by adding 50 µl/well of diluted DON monoclonal antibodies (clone 4 or 22) in PBS kindly provided by C. Maragos (USDA). After shaking and incubation for 1 h, 100 µl/well of Sec Ab-HRP was added and incubated for another 1 h. Then, 100 µl/well of TMB substrate solution was added. The reaction was stopped after 15 min with 2 M sulphuric acid (50 µl/well), and the absorbance at 450 nm was measured by a Bio-Rad 550 microplate reader (Richmond, CA, USA).

**Immunisations**

To obtain antibody-producing B-lymphocytes against DON, 6–8-week-old Balb/C female mice (ethical approval according to ethical commission for animals (ECD) 10/08) were subjected to an injection with the different DON immunogens emulsified with complete or incomplete Freund’s adjuvant. For each group of mice, 100 µg of the DON immunogen was administered. Once the mice had reached a sufficient titre, cell fusion of mouse spleen cells and myeloma cells (NSO cells) was performed. Polyethylene glycol (PEG) 1500 was added as fusing reagent and hypoxanthine, aminopterine and thymidine (HAT) for the selection of the fused cells. The cells were distributed into 96-well culture plates and cultured in a humidified, 37°C, 5% CO₂ incubator. Culture supernatant was screened by indirect ELISA to determine the positive hybridomas producing antibody against DON. These hybridomas were further screened for the production of the target antibody and subcloned by limiting dilution.
Characterisation of monoclonal antibodies

Checkerboard assays, in which antibodies were titrated against various amounts of coating antigens, were conducted to select appropriate working concentrations for evaluation of assay sensitivities to DON. Standard competitive curves were obtained by plotting relative absorbance (ratio of absorbance measured at the standard concentration and zero concentration: $B/B_0 \times 100\%$) against the logarithm of analyte concentration. IC$_{50}$ values (i.e., analyte concentrations at which the maximum absorbances were inhibited by 50%) were determined to assess the assay sensitivity. To evaluate the specificity or assay selectivity of the antibody, a set of DON analogues were utilised to perform cross-reactivity studies. The IC$_{50}$ of each tested compound was based on its corresponding competitive curve. Cross-reactivity (CR) values were calculated according to the following equation: $CR \% = \frac{IC_{50} \ (DON) \times 100\% \ (analogue)}{IC_{50} \ (analogue)}$.

Results and discussion

Characterisation of immunogens

When the desired synthesis products were obtained based on the exact mass measurements performed by direct injection into the Quattro Premier XE mass spectrometer or the LCT Premier XETM TOF mass spectrometer, further coupling to BSA and OVA or HRP was performed. The synthesised immunogens were characterised by competitive ELISA using reference DON monoclonal antibodies (clone 4 or 22, USDA). If the $B_0$ value was equal to or higher than 1 and decreasing $B$ values were obtained when using increasing standard DON concentrations, the testing conjugates were confirmed and used for immunisation or further ELISA experiments.

Determination of antisera titres by using indirect ELISA

Antisera titres were determined by an indirect homologous ELISA using the immunising hapten coupled to OVA instead of BSA as coating antigen (Guo et al. 2014). By using the same format, the cross-reactivity against OVA was determined as well. The best antisera titres were obtained for the mice injected with DON-BSA and DON-CC-BSA. For DON-3,15-HG-BSA, high antisera titres were found, but the cross-reactivity against OVA was high. It was concluded that antibodies were probably formed against the protein instead of the target DON. This could be explained by the presence of two linkers on the DON molecule, which makes it less free for activation of the immune system of the mouse. For DON-CMO-BSA, DON-CPI-BSA, DON-azido-BSA and DON-cyclic peptide-BSA, a relatively lower titre, but no cross-reactivity against OVA was determined. When the ELISA response reached a plateau phase using a high serum dilution, the mice were sacrificed by cervical dislocation.

Hybridoma selection and subcloning

By screening and subcloning of the hybridomas, finally, a total of three different anti-DON monoclonal antibodies were obtained. Corresponding to the derived antisera titres, one DON-CC-BSA mouse produced the 13H1 monoclonal antibody, while the 10H10 and 2A9 monoclonal antibodies were produced by two different DON-BSA mice. Previous statement about the immunogenic importance of the C8 carbonyl function in the DON molecule was confirmed as no anti-DON monoclonal antibodies were derived using immunogens with a linker coupled to the C8 carbonyl function (Usleber et al. 1991).

Characterisation of monoclonal antibodies

The characterisation of monoclonal antibodies 13H1 and 10H10 by direct ELISA is described in detail by Guo et al. (2014). The 13H1 and 10H10 monoclonal antibody showed sensitivity values of 2.13 and 0.022 µg ml$^{-1}$, respectively. For the 13H1 monoclonal antibody the highest cross-reactivity was observed for 15-ADON (CR = 131%) and HT-2 toxin (CR = 187%). Monoclonal antibody 10H10 showed the highest cross-reactivity for 3-ADON (CR = 147%) and 15-ADON (CR = 65%). Characterisation of 10H10 was also repeated by indirect competitive ELISA using DON-OVA (4 µg ml$^{-1}$) and DON-CC-OVA (4 µg ml$^{-1}$) coating and DON, 3-ADON and 15-ADON for competition. This comparison between direct and indirect ELISA is illustrated in Table 1. The sensitivity of the antibody towards DON and 3-ADON measured by indirect ELISA is approximately half (DON-OVA coating) and 1/5 (DON-CC-OVA coating) of the value measured by direct ELISA. For 15-ADON, the sensitivity decreased at least ten times when using indirect ELISA. When looking to the CR$_{molar}$ values for direct ELISA and indirect ELISA with DON-OVA coating, the same cross-reactivity is seen for 3-ADON, but the cross-reactivity towards 15-ADON lowered 6.7 times when using indirect ELISA. It can even be concluded that the 10H10 antibody shows only cross-reactivity to 3-ADON when using indirect ELISA with DON-OVA coating. When using an indirect ELISA format with DON-CC-OVA coating, the cross-reactivity against 3-ADON increased three times and the cross-reactivity against 15-ADON decreased four times compared to the direct ELISA format. So, the characterisation of the monoclonal antibody depends on the type of ELISA and the coating antigen used.

Characterisation of monoclonal antibody 2A9 was performed using direct ELISA and the standard curve using 3-ADON standard for competition is shown in Figure 4. The curve represents the relative absorbance
values (B/B₀) of the ELISA experiment performed on 3 consecutive days. A very sensitive anti-3-ADON antibody was developed with an IC₅₀ value of 0.38 ng ml⁻¹. Setting the monoclonal antibody activity for 3-ADON as 100%, the cross-reactivity values for DON, 15-ADON and DON-3-G (Table 2) were determined as 0.188%, 0.088% and 1.498% and can be considered as negligible. In comparison with other previously reported monoclonal antibodies for 3-ADON, the newly developed clone 2A9 is likely the most sensitive and specific antibody to 3-ADON (Casale et al. 1988; Maragos & McCormick 2000; Maragos et al. 2006; Baumgartner et al. 2010).

Conclusions

By using a three-series synthesis strategy for the development of specific monoclonal antibodies against DON, previous statements were confirmed and new conclusions could be made. The place of the linker on the DON molecule is of importance for the immunogenic response. When a linker is positioned on the C3 or C15 of DON for the synthesis of an immunogen, the produced antibody shows higher cross-reactivity against 3-ADON or 15-ADON, respectively. When coupling a linker to the carbonyl C8 of DON, no high anti-DON immune response was observed. This emphasises the immunogenic importance of the C8 carbonyl function. The size of the linker does not seem to influence the immunogenicity of the injected conjugate. The kind of linker has an influence on the electronic configuration of the DON molecule and therefore on the antigenic determinant of DON which is available for the immune system of the mouse.

Three different monoclonal antibodies were developed. One of them (2A9) can be called an anti-3-ADON monoclonal antibody, because of its very sensitive and specific characteristics towards 3-ADON. The second 10H10 monoclonal antibody is a broad specific antibody against DON and its acetylated derivatives 3-ADON and

Table 1. Comparison between direct and indirect ELISA for 10H10 antibody characterisation.

| Compound  | Direct ELISA | IC₅₀ (µg ml⁻¹) | CR (%) | IC₅₀ molar (nmol ml⁻¹) | CR molar (%) |
|-----------|-------------|----------------|--------|------------------------|-------------|
| DON       | 0.022       | 100            | 0.074  | 100                    |
| 3-ADON    | 0.015       | 147            | 0.044  | 167                    |
| 15-ADON   | 0.034       | 65             | 0.101  | 74                     |
| DON-OVA   | 0.040       | 100            | 0.135  | 100                    |
| 3-ADON    | 0.028       | 142            | 0.083  | 162                    |
| 15-ADON   | 0.421       | 9              | 1.250  | 11                     |

Table 2. Comparison of cross-reactivity results between clone 2A9 and other DON-MAbs.a

| Compound | Clone 2A9 direct ELISA | Mab 1–6.2.6 (1) indirect ELISA | USDA Clone 22 (4) direct ELISA |
|----------|------------------------|-------------------------------|-------------------------------|
|          | IC₅₀ (ng ml⁻¹) | CR (%) | IC₅₀ (ng ml⁻¹) | CR (%) | IC₅₀ (ng ml⁻¹) | CR (%) |
| 3-ADON   | 0.38       | 100    | 1.70          | 100    | 2.88         | 100    |
| DON      | 202.14     | 0.188  | 15.80         | 10.7   | 18.20        | 15.8   |
| 15-ADON  | 431.79     | 0.088  | 68.90         | 2.4    | 558.00       | 0.52   |
| DON-3-G  | 25.36      | 1.498  | n.a.          | –      | n.a.         | –      |

Notes: aHerein, CR (%) = [IC₅₀ (3-ADON)/IC₅₀ (analogue)] × 100%.
For comparison, the results from reference (4) by Maragos & McCormick (2000) were competitive direct ELISA for clone 22; the results from reference (1) by Maragos et al. (2006) were competitive indirect ELISA for Mab 1–6.2.6.
15-ADON when using it in a direct ELISA format. By using an indirect ELISA format with DON-OVA or DON-CC-OVA coating, the selectivity of this antibody changes resulting in only cross-reactivity against 3-ADON. When combining the 2A9 and 10H10 antibodies in an indirect ELISA format, it is possible to measure the sole DON contamination.

Therefore, we conclude that it is possible to produce specific antibodies against trichothecene mycotoxins such as 3-ADON by synthesising the proper immunogen with the suitable linker and screening the hybridomas carefully. By changing the ELISA format and/or coating antigen, it is possible to influence the selectivity and cross-reactivity of the monoclonal antibody.

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References
Abuchowski A, Van Es T, Palczuk N, Davis F. 1977. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem. 252:3578–3581.
Abuknesha R, Griffith H. 2005. Generation of antiserum to Irgarol 1051 and development of a sensitive enzyme immunoassay using a new heterologous hapten derivative. Anal Bioanal Chem. 381:233–243.
Baumgartner S, Führer M, Kriska R. 2010. Comparison of monoclonal antibody performance characteristics for the detection of two representatives of A- and B-trichothecenes: T-2 toxin and deoxynivalenol. World Mycotoxin J. 3:233–238.
Berthiller F, Dall’asta C, Corradi R, Marchelli R, Sulyok M, Kriska R, Adam G, Schuhmacher R. 2009. Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. Food Addit Contam: Part A. 26:507–511.
Burkin A, Kononenko G, Soboleva N, Zotova E. 2000. Preparation of conjugated antigens based on zearalenone carboxymethylxime and their use in enzyme immunoassay. Appl Biochem Microbiol. 36:282–288.
Casale WL, Pestka JJ, Hart LP. 1988. Enzyme-linked immuno-absorbent assay employing monoclonal antibody specific for deoxynivalenol (vomitoxin) and several analogs. J Agr Food Chem. 36:663–668.
Dai XD, Su Z, Liu JO. 2000. An improved synthesis of a selective α,β-3-integrin antagonist cyclo(-RGDFK-). Tetrahedron Lett. 41:6295–6298.
Danicke S, Goyarts T, Doll S, Grove N, Spolders M, Flachowsky G. 2006. Effects of the Fusarium toxin deoxynivalenol on tissue protein synthesis in pigs. Toxicol Lett. 165:297–311.
De Boever M, Di Mavungu JD, Landschoot S, Audenaert K, Eeckhout M, Maene P, Haesaert G, De Saeger S. 2012. Natural occurrence of mycotoxins and their masked forms in food and feed products. World Mycotoxin J. 5:207–219.
De Smet D, Monbaliu S, Dubruel P, Van Peteghem C, Schacht E, De Saeger S. 2010. Synthesis and application of a T-2 toxin immobilized polymer. J Chromatogr A. 1217:2879–2886.
Dijkstra I, Rijnders AY, Soede A, Dechesne AC, van Esse GW, Brouwer AJ, Corstens FHM, Boerman OC, Rijkers DTS, Liskamp RMJ. 2007. Synthesis of DOTA-conjugated multivalent cyclic-RGD peptide dendrimers via 1,3-dipolar cycloaddition and their biological evaluation: implications for tumor targeting and tumor imaging purposes. Org Biomol Chem. 5:935–944.
Döll S, Dänicke S. 2011. The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. Prev Vet Med. 102:132–145.
Guo Y, Sanders M, Galvita A, Heyericck A, Deforce D, Bracke M, Eremin S, De Saeger S. 2014. Heterologous screening of hybridomas for the development of broad-specific monoclonal antibodies against deoxynivalenol and its analogues. World Mycotoxin J. 7:257–265.
Horak J, Hofer S, Lindner W. 2010. Optimization of a ligand immobilization and azide group endcapping concept via “Click-Chemistry” for the preparation of adsorbents for antibody purification. J Chromatogr B Anal Technologies Biomed Life Sci. 878:3382–3394.
Ikuina Y, Amishiro N, Miyata M, Narumi H, Ogawa H, Akiyama T, Shiotzu Y, Akinaga S, Murakata C. 2003. Synthesis and antitumor activity of novel O-carbamoylmethylxime derivatives of radicicol. J Med Chem. 46:2534–2541.
JECFA. Joint FAO/WHO Expert Committee on Food Additives. 2011. Safety evaluation of certain contaminants in food. Prepared by the seventy-second meeting of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series 63.
Kohno H, Yoshizawa T, Fukugi M, Miyoshi M, Sakamoto C, Hata N, Kawamura O. 2003. Production and characterization of monoclonal antibodies against 3,4,15-triacetylnivalenol and 3,15-diacetyldesoxyxovalenol. Food Agric Immunol. 15:243–254.
Krska R, Baumgartner S, Josephs R. 2001. The state-of-the-art in the analysis of type-A and -B trichothecene mycotoxins in cereals. Fresenius J Anal Chem. 371:285–299.
Liskamp RM, De Smet D, Monbaliu S, Dubruel P, Van Peteghem C, Schacht E, De Saeger S. 2010. Synthesis and application of a T-2 toxin immobilized polymer. J Chromatogr A. 1217:2879–2886.
Maragos CM, Li L, Chen D. 2012. Production and characterization of two representatives of A- and B-trichothecenes: T-2 toxin and deoxynivalenol. World Mycotoxin J. 3:233–238.
M, Eremin S, De Saeger S. 2014. Heterologous screening of hybridomas for the development of broad-specific monoclonal antibodies against deoxynivalenol and its analogues. World Mycotoxin J. 7:257–265.
S., De Saeger S. 2010. Synthesis and application of a T-2 toxin immobilized polymer. J Chromatogr A. 1217:2879–2886.
Dijkstra I, Rijnders AY, Soede A, Dechesne AC, van Esse GW, Brouwer AJ, Corstens FHM, Boerman OC, Rijkers DTS, Liskamp RMJ. 2007. Synthesis of DOTA-conjugated multivalent cyclic-RGD peptide dendrimers via 1,3-dipolar cycloaddition and their biological evaluation: implications for tumor targeting and tumor imaging purposes. Org Biomol Chem. 5:935–944.
Döll S, Dänicke S. 2011. The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. Prev Vet Med. 102:132–145.
Guo Y, Sanders M, Galvita A, Heyericck A, Deforce D, Bracke M, Eremin S, De Saeger S. 2014. Heterologous screening of hybridomas for the development of broad-specific monoclonal antibodies against deoxynivalenol and its analogues. World Mycotoxin J. 7:257–265.
Horak J, Hofer S, Lindner W. 2010. Optimization of a ligand immobilization and azide group endcapping concept via “Click-Chemistry” for the preparation of adsorbents for antibody purification. J Chromatogr B Anal Technologies Biomed Life Sci. 878:3382–3394.
Ikuina Y, Amishiro N, Miyata M, Narumi H, Ogawa H, Akiyama T, Shiotzu Y, Akinaga S, Murakata C. 2003. Synthesis and antitumor activity of novel O-carbamoylmethylxime derivatives of radicicol. J Med Chem. 46:2534–2541.
JECFA. Joint FAO/WHO Expert Committee on Food Additives. 2011. Safety evaluation of certain contaminants in food. Prepared by the seventy-second meeting of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series 63.
Kohno H, Yoshizawa T, Fukugi M, Miyoshi M, Sakamoto C, Hata N, Kawamura O. 2003. Production and characterization of monoclonal antibodies against 3,4,15-triacetylnivalenol and 3,15-diacyldeoxyxovalenol. Food Agric Immunol. 15:243–254.
Krska R, Baumgartner S, Josephs R. 2001. The state-of-the-art in the analysis of type-A and -B trichothecene mycotoxins in cereals. Fresenius J Anal Chem. 371:285–299.
Maragos C, Busman M, Sugita-Konishi Y. 2006. Production and characterization of a monoclonal antibody that cross-reacts with the mycotoxins nivalenol and 4-deoxynivalenol. J Food Addit Contam. 23:816–825.
Maragos CM, Li L, Chen D. 2012. Production and characterization of a single chain variable fragment (scFv) against the mycotoxin deoxynivalenol. Food Agric Immunol. 23:51–67.
Maragos CM, McCormick SP. 2000. Monoclonal antibodies for the mycotoxins deoxynivalenol and 3-acetyl-deoxynivalenol. Food Agric Immunol. 12:181–192.
Mills C, Alcock S, Lee H, Morgan M. 1990. An enzyme-linked immunosorbent assay for deoxynivalenol in wheat, utilizing novel hapten derivatization procedures. Food Agric Immunol. 2:109–118.
Pestka JJ. 2007. Deoxynivalenol: toxicity, mechanisms and animal health risks. Anim Feed Sci Technol. 137:283–298.
Pinton P, Tsybulskyy D, Lucioli J, Laffitte J, Callu P, Lyazhri F, Grosjean F, Bracarense AP, Kolf-Clauw M, Oswald IP. 2012. Toxicity of deoxynivalenol and its acetylated derivatives on the intestine: differential effects on morphology, barrier function, tight junction proteins, and mitogen-activated protein kinases. Toxicol Sci. 130:180–190.
Sinha R, Savard M, Laur R. 1995. Production of monoclonal antibodies for the specific detection of deoxynivalenol and 15-acetyldeoxynivalenol by ELISA. J Agr Food Chem. 43:1740–1744.
Usleber E, Maerlhuber E, Dietrich R, Terplan G. 1991. Direct enzyme-linked immunosorbent assays for the detection of the 8-ketotrichothecene mycotoxins deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol in buffer solutions. J Agr Food Chem. 39:2091–2095.
van Dongen SFM, Teeuwen RLM, Nallani M, van Berkel SS, Cornelissen JJLM, Nolte RJM, van Hest JCM. 2009. Single-step azide introduction in proteins via an aqueous diazo transfer. Bioconj Chem. 20:20–23.
Xiao H, Clarke JR, Marquardt RR, Frohlich AA. 1995. Improved methods for conjugating selected mycotoxins to carrier proteins and dextran for immunoassays. J Agr Food Chem. 43:2092–2097.