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Murine immunomodulation by low-dose dietary deoxynivalenol and improved detection methods for deoxynivalenol

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Murine immunomodulation by low-dose dietary deoxynivalenol and improved detection methods for deoxynivalenol

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ABSTRACT

Deoxynivalenol (DON) is a mycotoxin naturally occurring in worldwide grain supplies and has caused gastrointestinal disease and immunotoxicity in animals. Methods were optimized for analysis and screening of food samples for DON. Immunomodulatory effects of low levels ($\leq$ 2 mg/kg) dietary DON and acute exercise stress was investigated in BALB/c mice as a model for potential interaction in humans. Caffeine (CAF) was found to be a suitable internal standard for analytical detection of DON in wheat samples using high performance liquid chromatography (HPLC) with ultraviolet detection. Peak area response ratios DON: CAF were adequately precise over multiple runs and a range of spiked samples (0.25 – 6.0 ppm DON). This method will allow analysis of samples using an easily accessible, stable, and inexpensive compound that is not likely to be found in most samples of interest. A bioassay was used to screen for DON in the range of common contamination in a variety of food samples. Human K-562 erythroleukemia cells, modeling immune stem cells, were incubated with wheat and corn extract residues dissolved in complete media and cell proliferation was assessed by the MTS [dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] dye reduction assay. Samples containing DON ranged in concentrations from 204 to 3792 ng DON/g food which strongly correlated with HPLC analysis. This sensitive bioassay may serve as an inexpensive low-tech alternative for screening food samples or products of DON detoxification studies. We hypothesized that acute exercise stress would exacerbate immunosuppressive effects of sub-acute exposure to dietary deoxynivalenol (DON). Male BALB/c mice were fed 0 or 2 mg DON/kg for 14 days (n=12 per dose), then half of each treatment group were exercised to fatigue on a treadmill. Only the non-exercised DON-fed mice showed significant splenocyte proliferation suppression with increased IL-4 expression in response to mitogen stimulation. Antibody response to T-dependent antigen (sheep red blood cells) was significantly less for exercised DON-fed mice with increased IL-2 expression. Serum corticosterone levels were significantly higher for both exercised groups. Our hypothesis was confirmed with respect to T-cell dependent antibody production, but not for splenocyte proliferation. Exercise stress protected against DON-mediated suppression of splenocyte proliferation, perhaps mediated by induction of elevated stress hormones counteracting cytokine expression alterations of

DON. This interaction was investigated further with BALB/c male mice fed 0, 1 or 2 ppm DON for 28 days then half of each treatment group was exercised to fatigue on a treadmill. Exercise was inhibitory for IL-4, NK cytotoxicity, spleen: body weight ratio, blood lymphocytes, hemoglobin, hematocrit but caused elevated blood neutrophils. Dietary 2 ppm DON inhibited weight gain, red blood cell numbers and hematocrit but increased feed intake. DON fed at 1 ppm stimulated NK cytotoxicity, PFCs, and spontaneous IFN-γ secretion. Blood lymphocytes were suppressed by DON feeding independent of dose. This is the first report of *in vivo* immunostimulatory effects of sub-chronic low dose DON feeding. DON fed at 1 ppm seemed to promote Th1 responses *in vivo*, most likely through differential cell signal transduction affecting cytokine production, which may be abrogated by acute exercise stress. Apparent immunological hormesis of feeding 1 ppm DON deserves further investigation.

CHAPTER 1. GENERAL INTRODUCTION

Research Questions

The work discussed in this dissertation originated from interests in food safety. Previous job duties had included inspection of food supplies with a familiarization of toxicological and microbiological screening of foods for mass consumption. Although DON work had not been conducted in our lab previously, this mycotoxin had some characteristics that warranted further investigation. After a cursory review of current knowledge of DON, it became apparent that this mycotoxin occurred in a wide variety of staple grains and screening for its presence in these foods was sporadic or non-existent. Human illnesses had occurred that appeared to be caused by DON exposure in foods, usually as epidemics although clear linkage between the mycotoxins presence in foods and human illness have yet to be confirmed. Though grain supplies in the United States are screened for DON, at industry discretion, foreign procured food would be less likely to be tested.

Our initial interest in DON research was development of high throughput low-technology screening tools for field use. There are ELISA kits for DON on the market but may be too sophisticated and expensive for use in under-developed countries. Prior to screening assay development, a reference analytical method was needed. High performance liquid chromatography methods were published and peer-reviewed but required two analyses per sample as no internal standards for this method had been developed. We felt that an internal standard method would simplify the analytical method as well as reduce the volume of hazardous wastes produced.

Screening assay development as a primary goal of research continued but numerous methods were contemplated. Immunological effects of DON had been published as well as several bioassays. Bioassays had been developed as tools to compare absolute toxicity of several mycotoxins but most cells used were not derived from nor modeled DON sensitive tissue *in vivo*. K-562 cells had been tested and found sensitive to purified DON in media within the range of DON sensitivity of human immune cells. Cereal sample extracts had been screened for DON toxicity on fibroblast cell lines. We hypothesized that these assays could be combined and a bioassay that was relative to known *in vivo* DON toxicity would be useful to assess detoxification strategies and metabolic products of DON in the future.

A large gap of knowledge in toxicological research was *in vivo* responses to toxin interactions. Granted, human research is difficult to conduct in this area and effects of many confounding factors must be considered. Controlled animal experiments may provide evidence that could focus or deemphasize the importance of confounding factors. Evidence exists for immunological effects of DON on the immune system, however limited systematic studies have been reported and no epidemiological studies in humans. Swine are the most sensitive species to DON and evidence suggests similar bioavailability for most monogastric animals. While not lethal to pigs, DON causes vomiting and poor growth performance at low dietary levels. Limited immunological assays have been conducted in pigs, due to the lack of reagents and expense of these studies. Mouse studies have the advantage of a large range of reagents available, adequate samples sizes to validate findings, and historically comparable immunological responses to humans.

Exercise stress in mice as a model for human stress has been established. Stress can cause immunomodulation in humans and animals depending on the specific stressor duration, intensity, and frequency of exposure. Acute exercise stress is simple to induce in mice and has been comparable with human responses to similar stresses. As little work has been conducted investigating interactions of xenobiotic exposure and other environmental stressors, we hypothesized that dietary DON (doses causing minimal immunosuppression in mice) would exacerbate immunosuppressive effects of acute exercise stress. We further wondered about mechanisms of immunomodulation by these two agents including evaluations of effector molecules such as cytokines. Research reported here indicates importance of conducting epidemiologic studies to determine actual human exposures to ubiquitous mycotoxin food contamination and potential for previously unidentified interactions of food contaminants with environmental stressors and implications for immunomodulation.

Dissertation Organization

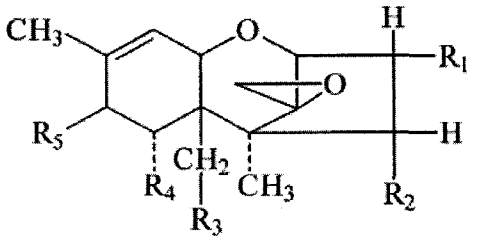
This document contains a general literature review, including the most relevant information to the research questions proposed. Four manuscripts for publication are

included as separate chapters. All references cited are listed in a separate section at the end of the dissertation.

CHAPTER 2. LITERATURE REVIEW

General Information

Deoxynivalenol is a trichothecene mycotoxin. There are about 180 trichothecenes but only a handful are significant in human and animal health. The first report of the structure of DON and its identification as the toxic principal from grain known to produce vomiting in swine was by Vesonder et al. (1973). Following organic solvent extraction and purification by thin layer and column chromatography, a single toxic fraction was isolated and analyzed by mass spectroscopy to determine the molecular weight (296.16) with an empirical formula of $C_{15}H_{20}O_6$. Based on infrared and nuclear magnetic resonance spectra a chemical structure was proposed which was related but unique from already identified trichothecenes T-2 toxin and fusarenon-X. The molecular structure of DON and other related trichothecenes is illustrated below (Figure 2.1).



Trichothecene	R1	R2	R3	R4	R5
Deoxynivalenol	OH	H	OH	OH	=O
3-Acetyldeoxynivalenol	OAC	H	OH	OH	=O
15-Acetyldeoxynivalenol	OH	H	OAC	OH	=O
Fusarenon-X	OH	OAC	OH	OH	=O
Nivalenol	OH	OH	OH	OH	=O

Figure 2.1. Trichothecene skeleton and sidechains of selected mycotoxins.

Biosynthesis –

Fusarium species of fungi are numerous and ubiquitous in the environment and can be pathogenic to common staple crops such as wheat, corn and barley. These fungi can

produce secondary metabolites such as mycotoxins. Mycotoxin production by these organisms has been known for a long time but more recently the connection between fungal species, mycotoxin(s) produced, and environmental conditions favorable for biosynthesis of these molecules has been elucidated. Deoxynivalenol is produced by three *Fusarium* species: *graminearum, pseudograminearum,* and *culmorum*. Miller et al. (1991) cultured *Fusarium graminearum, culmorum*, and *crookwellense* collected from China, Mexico, and the Netherlands. Each isolate was associated with plant pathology in cereals and was grown in liquid media and on rice to characterize the secondary metabolites. The metabolites generally fell into two classes; major metabolites, including trichothecenes, that were characteristic for the specific strain, and minor metabolites of trichothecene-related compounds common to many fusaria. From gas chromatography/mass spectroscopic analysis of the metabolites, chemotypes were proposed based on chemical structure of mycotoxins produced. *Fusarium graminearum* and *culmorum* had overlapping chemotypes since they had similar profiles. Chemotype IA produced DON and 3-acetyldeoxynivalenol (3-ADON) and chemotype IB produce DON and 15-acetyldeoxynivalenol (15-ADON). Chemotype IB is the prominent chemotype in North America. Asian isolates tend to be chemotype IA indicating genetic variability in biosynthetic pathway enzymes. Other chemotypes of *F. graminearum* are II, which produces nivalenol, and IIA producing nivalenol and 4-acetylnivalenol. From this work, a systematic approach to classifying toxigenic *Fusarium* species, based on the specific mycotoxins produced, complementing the taxonomic classification. Genetic variability as a major contributor to mycotoxin biosynthesis potential was more defined.

Much effort has been focused on the genetic influence on trichothecene biosynthesis since this information could elucidate a target for genetically modified control. All of the biosynthetic pathway enzymes have not been determined but Moss and Thrane (2004) indicated that the production of trichothecenes was not trivial since it required a substantial energy commitment of at least 9 acetyl CoA, 6 NADPH, 9 ATP molecules, and cytochrome P450 activity. Little was known about the purpose of mycotoxin production by the fungi. Lutz et al. (2003) determined a specific beneficial effect of DON biosynthesis. They inoculated maize leaf tissue with DON-producing strains of *F. culmorum*, a genetically modified *F. graminearum* DON-nonproducer, and *Trichoderma,* an antagonistic fungus

which can competitively colonize field residues and produces chitinase and other fungal cell wall-degrading enzymes. Once confirmation studies were conducted for DON production in the proposed *in vitro* system, it was determined that DON repressed a specific chitinase gene (*nag1*) in the *Trichoderma.* They reported that this was the first evidence of specific genetic alteration on an antagonistic organism although many variables influenced the effect on the *Trichoderma* including the age and cultivar of the residue. Since this work was conducted on the bench many questions still exist about similar effects occurring in the field and possible futility of continued biocontrol strategy development. Even with a great deal of effort toward control of *Fusarium* infection on staple grains, trichothecene contamination occurred globally although sporadically depending on environmental conditions.

Edwards (2004) reviewed the environmental conditions found to favor mycotoxin accumulation in food crops. Risk factors associated with increased DON accumulation were: the crop grown the preceding two years particularly grain maize the previous year; minimum tillage of the soil was more likely to be associated with DON contamination when compared to deeper tillage such as ploughing; application of nitrogen fertilizers; application of fungicides (azoxystrobin increased DON contamination in field trials); and herbicide (glyphosate) application. Biological and insect control has shown promise in the laboratory environment but little or no progress has been made in the field. Edwards further expressed the evidence for synergistic interactions of risk factors for DON production as reported by Obst et al. (2000). Risk factors for DON contamination of wheat were crop rotation after maize planting, minimum tillage after maize planting, susceptible wheat variety, application of strobilurin fungicide, and warm wet weather during flowering. Presence of one of these risk factors increased the risk of DON accumulation by a factor of three whereas presence of four risk factors increased risk 56 fold. This information was helpful for attempting to educate the wheat producers on practices to reduce mycotoxin risk. DON was the only mycotoxin reviewed and production of other mycotoxins may occur even under conditions that would be predicted to reduce DON since changes in environmental conditions seem to favor production of specific mycotoxins. Much more research will need to be conducted before control of mycotoxin production in the field is successful.

DON may be produced by *Fusarium* spp. as a secondary metabolite, synthesis not required for survival or growth, along with other closely related trichothecenes. Taxonomic classification of *Fusarium* sp. was supplemented with chemotype designation based on specific trichothecenes production (Miller et al. 1991). Even though all biosynthetic pathways have not been determined, cellular energy resources are required for trichothecene production (Moss and Thrane, 2004) and they may serve to promote competitive advantage over antagonistic fungi on crop residues (Lutz et al., 2003). Conditions that increased risk of a DON outbreak in a crop were associated with tillage practices, environmental conditions, and crop rotation (Edwards, 2004). There are still knowledge gaps in the finer points of trichothecene production from *Fusarium* that do not allow accurate predictions of crop contamination outbreaks. Work not reported here, has been published concerning crop improvements to resist fungal infection but the fungi mutate rapidly and even if resistance could be effectively engineered into a crop, the resistant cultivar may only be useful in a fairly small region. Further work on the biosynthetic pathway may lead to identification of upstream, highly conserved non-essential foci that will provide effective generalized crop resistance.

Worldwide occurrence

Outbreaks of contamination occur regularly in worldwide food supplies regardless of any control efforts for natural mycotoxin contamination. Since analytical methods have become more prevalent, sensitive, and specific, food surveillance for mycotoxin contamination has become more common. The following is a table of some of the surveys that have been conducted with information on method used and other contaminants found.

Table 2.1. Worldwide Mycotoxin Surveys

Country	Food/Feed type	DON mg/kg	# positive/ # tested	Other mycotoxins detected	Detection Method (limit of detection)	References
China	Raw corn Cornmeal Cooked pancakes	<0.5 – 2.7 <0.5 – 1.6 <0.5 – 1.5	7/12 8/13 4/14	FB1, FB2, FB3, 15ADON	GC/MS (0.5 mg/kg)	Groves et al. (1999)
China	Wheat Barley Corn	0.016 – 51.45 0.132- 3.521 0.256 – 21.200	8/8 2/2 2/2	NIV, 3ADON, ZEA NIV, ZEA NIV, ZEA, FB1, FB2, FB3, 15ADON	GC/MS (trichothecenes 0.01 mg/kg) HPLC (fumonisins 0.05 mg/kg)	Li et al. (1999)

Table 2.1. (continued)

Nepal	Corn grain Corn flour Cornflakes	nd – 11.00 nd – 3.00 nd	10/58 1/8 0/2	Fumonisins Fumonisins	Immunoassay (1.0 mg/kg) Quantitative fluorometry (1.0 mg/kg)	DesJardins et al. (2000)
Portugal	Bran cereal Wheat flakes Wheat & fruit cereal	<0.1 - >5.001 <0.1 – 5.000 <0.1 – 5.000	16/24 16/20 32/44	–	HPLC (0.1 mg/kg)	Martins and Martins (2001)
Germany	White wheat flour White wheat flour Whole wheat flour	0.239 ± 0.228 0.234 ± 0.215 0.404 ± 0.427	28/28 12/13 19/19	NIV, ZEA NIV, HT-2, ZEA NIV, 3ADON, 15ADON, HT-2, T-2, ZEA	GC/MS (0.002 – 0.012 mg/kg)	Schollenberger et al. (2002)
Germany	Wheat flour Wheat bread Noodles Rice Oats & products Corn & products	0.394 mean 0.956 (90th %) 0.125 mean 0.247 (90th %) 0.275 mean 1.149 (90th %) 0.058 mean 0.024 mean 0.034 mean	59/60 127/141 61/67 14/26 12/23 25/36	discussed but not reported	GC/MS (0.007 mg/kg)	Schollenberger et al. (2003)
Canada	Oat-based infant cereals Barley-based infant cereal Soy based infant cereal Multi-grain infant cereals Rice-based infant cereals Teething biscuits Soy formulas Creamed corn	0.052 mean 0.090 max. 0.260 mean 0.980 max. 0.116 mean 0.240 max. 0.116 mean 0.400 max. nd 0.060 mean 0.120 max. nd nd	33/53 29/50 8/8 62/86 0/9 18/24 0/1 0/6	OTA, ergot alkaloids ZEA, OTA, ergot alkaloids ZEA, OTA, FB ZEA, OTA, FB, ergot ZEA, OTA, FB ZEA, OTA, FB, ergot nd nd	GC/MS (limit of quantification DON 0.020 mg/kg)	Lombaert et al. (2003)
United States	Durum wheat	nd – 23.0	123 tested	15ADON (nd – 0.8) NIV nd	GC (LOD not reported)	Manthey et al. (2004)
United States	Hard winter wheat Soft winter wheat Hard spring wheat Soft white wheat Mixed wheat Barley Malting barley	0.8 mean 7.6 max. 1.4 mean 14.6 max 3.7 mean 18.4 max. 0.1 mean 0.7 max. 2.3 3.0 mean 14.0 max. 9.0 mean 25.8 max.	94/194 50/59 180/201 8/28 1/1 79/118 29/29	not reported	ELISA (0.5 mg/kg)	Trucksess et al. (1995)

Table 2.1. (continued)

Italy	Cereals, whole meals, flours	0.065 median (0.065 – 0.930 range)	111/111	FB1, FB2	GC/ECD (0.007 mg/kg)	Cirillo et al. (2003)
	Breads	0.046 median (0.007 – 0.270)	19/24	FB1, FB2		
	Durum wheat pasta	0.019 median (0.009 – 0.077)	7/17	FB2		
	Breakfast cereals	0.023 median (0.012 – 0.047)	9/14	FB1, FB2		
	Biscuits	0.040 median (0.016 – 0.150)	16/24	FB1, FB2		
	Baby & infant foods	0.035 median (0.007 – 0.166)	7/12	nd		
Denmark	Wheat	0.144 mean 0.527 max.	75/88	NIV, HT-2, T-2, ZEA	GC-ECD (0.020 mg/kg)	Rasmussen et al. (2003)
	Rye	0.043 mean 0.257 max.	41/69	NIV, HT-2, T-2, ZEA		
	Durum wheat	1.155 mean 2.591 max.	33/33	NIV, T-2		
United Kingdom	Polenta	0.587 mean ± 0.135 sd	2/2	ZEA, FB1, FB2, FB3	GC/MS (0.050 LOQ)	FSA (2005)
	Corn cereals	0.227 mean ± 0.306 sd	15/44	ZEA, FB1, FB2, FB3, NIV		
	Snacks	0.157 mean ± 0.077 sd	19/38	ZEA, FB1, FB2, FB3, OTA		
United Kingdom	Cereals	0.138 mean ± 0.355 sd	42/60	3ADON, 15ADON, FUSX, NIV, T-2, HT-2, ZEA	GC/MS (0.010 LOQ)	FSA (2003)
	Cookies	0.033 mean ± 0.044 sd	49/60	NIV, ZEA		
	Bread	0.058 mean ± 0.062 sd	57/60	NIV, ZEA		
	Cakes	0.024 mean ± 0.015 sd	19/40	-		
	Flour	0.061 mean ± 0.091 sd	36/40	NIV		
	Polenta	0.175 mean ± 0.139 sd	7/8	15ADON, NIV, ZEA		
	Snacks	0.251 mean ± 0.269 sd	36/40	3ADON, 15ADON, NIV, ZEA		
	Corn flour	-	0/8	ZEA		
	Baby food	0.047 mean ± 0.044 sd	13/17	NIV, HT-2, ZEA		
	Snacks (2nd survey)	0.092 mean ± 0.073 sd	37/44	15ADON, NIV, ZEA		

This long list is not complete but provides a snapshot of the diversity of mycotoxins in foods to which humans were exposed.

Worldwide regulations

The regulation of mycotoxins worldwide was recently reviewed by the Food and Agricultural Organization of the United Nations (FAO 2004). Guidelines or regulation of DON was reported for 37 countries. DON levels allowed in human food range from zero tolerance in baby food (Belarus) to 2000 µg/kg in corn (Czech Republic). The Food and Drug (FDA) administration of the United States has an advisory level of 1000 µg/kg in

finished wheat products for human consumption. Figure 2.2 illustrates the range of tolerated levels in food for adult consumption and the number of countries using that guideline.

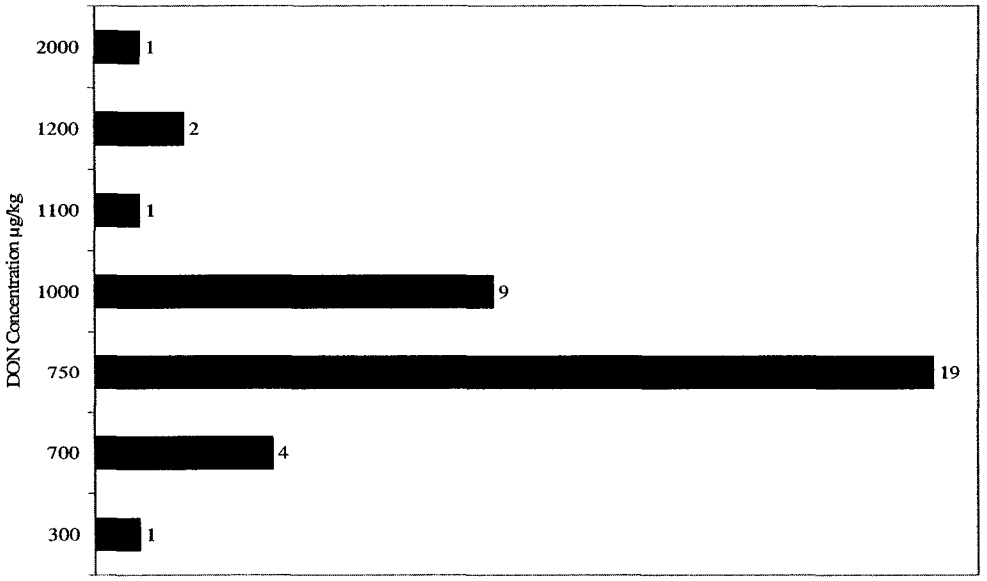


Figure 2.2. Regulatory levels of DON in human foods, with number of countries using each regulatory level

A complete listing of the countries and specific regulations for all mycotoxins regulated in foods and feeds is available (FAO, 2004).

Economic significance of contamination

The Council for Agricultural Science and Technology projected the annual cost of DON contamination in cereal grains in the United States (CAST 2003). The model included 727 samples of wheat and corn food and feeds from 1995-1997 FDA and private survey samples. Food losses were based on samples with > 1 ppm and feed losses where DON levels were greater than the advisory level for the intended species feed. From the survey samples, 6.9% of corn samples and 12.4% of wheat samples had DON contamination greater than 1 ppm. Both grains had annual output of 900 million bushel each per year. Wheat feed output was 275 million bushel with 1.8% exceeding advisory levels. Other costs considered were diverting crops for alternate uses, separation of contaminated from clean crops, and losses in livestock due to the current toxicity assessments. It was estimated that annual human food crop loss was $637 million, with $18 million lost feed crops and $2 million in livestock costs. The model assumed that all output loads were tested but most shipments were not thus

underestimation of losses was likely. Data on human toxicity was lacking so that no costs could be assigned. The authors were conscious of unknown factors and clearly indicated that these results were only a preliminary attempt. Regardless, the value of crop loss from DON contamination was substantial and efforts to remediate DON contamination are ongoing.

Resistance to processing

One of the difficulties when dealing with this natural food contaminant is its resistance to remediation through common food processing practices. Scott et al. (1983) were one of the first groups to assess DON retention in wheat from milling to baking. A naturally contaminated hard spring wheat sample, approximately 7.1 mg/kg DON as harvested, was cleaned and processed through a laboratory mill with fraction sample collection. The end product flour from each of duplicate lots was baked into three loaves by a standard bread recipe at 205° C for 30 minutes. Samples were analyzed by GC-electron capture and confirmation with GC-MS with method recovery of DON spiked samples 86 ± 7.8% (n = 3). The initial concentration of the wheat by the experimental methods was 4.62 mg/kg with a higher concentration occurring in the dockage, cleaning screenings (16.7 mg/kg), but this fraction was only 0.9% of total weight processed. The bran, shorts, feed flour and final flours had approximately equal concentrations (3.96 to 7.42 mg/kg) and the finished bread had at least 4.00 mg/kg. No statistical analysis was reported to compare the DON levels between the milling fractions or differences between analytical methods employed. This experiment revealed the potential for DON to continue through the food supply chain in common finished products.

DON retention in baked products was also conducted by El-Banna et al. (1983) investigating the effect of higher baking temperatures used to cook Egyptian bread. A naturally contaminated whole wheat flour (3.31 mg/kg) and spiked uncontaminated whole wheat flour was made into dough and fermented by a traditional recipe. Bread was baked at 350° C for 2 minutes. Samples were analyzed by GC-ECD and confirmed by GC-MS with recoveries of DON spiked into flour, fermented dough, and bread of 99.4 ± 3.4, 94.6 ± 3.7, and 96.1 ± 4.9% respectively. The experiment was replicated three times and comparisons were made between the two analytical methods used. Statistical analysis was not reported but the method seemed to improve over time as the DON recovery from spiked samples became

closer to 100% with smaller standard deviations in the final replication. They found no loss of DON through the fermentation or baking for naturally or spiked flours. Thus higher baking temperature did not affect the recovered concentration of DON, nor did the fermentation.

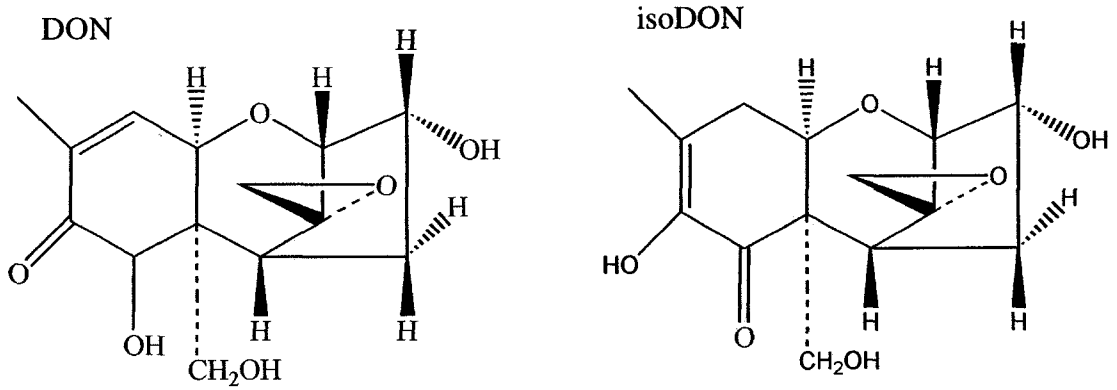


Figure 2.3. Chemical structures of deoxynivalenol and isodeoxynivalenol.

Greenhalgh et al. (1984) provided some evidence for a thermal conversion of DON to an isomer of DON (isoDON, Figure 2.3) as an explanation for disappearance of DON during some food production processes. IsoDON was synthesized and characterized through chemical acetylation and hydrolysis followed by thin-layer chromatographic purification and structural identification by ^{1}H NMR. Thermal treatment of purified DON in nitrogen at 160° C for 1 hour resulted in 65% loss of parent DON and 95% loss at 200°, with production of four new peaks as determined by mass spectroscopy. IsoDON was identified as one of the peaks, although it appeared to be a minor product and the other three peak's identification was not reported. When naturally contaminated wheat flour (0.5 mg DON/kg) and spiked wheat flour (0.1 and 3.0 mg DON/kg) were baked into bread, isoDON appeared in greater concentration in the crust than the crumb (estimated concentrations were not reported). It was stated that isoDON was present at 3 to 13% but it was not clear if this was a percent of the parent DON. The isomer was not present in the unbaked flours, but occurred in some wheat-based heat-processed breakfast cereals manufactured from naturally contaminated wheat. Validity of these results was questionable as no statistical analyses or replications were

reported. This study provided some evidence for a compound that may be produced from DON due to processing but it appeared to be minor and the previous two studies did not report a significant reduction of DON through bread baking. An issue that was not addressed was the difference in apparent substantial disappearance of DON during thermal processing of the purified compound versus the lack of similar disappearance in the foods. IsoDON production may not be relevant if it does not occur to a greater extent in foods where DON may be protected from transformation. Importance of production of this compound would be confirmed by toxicity assessment of the processed product.

Following an epidemic year for DON contamination in wheat Seitz et al. (1986) investigated the effects of four wheat cleaning methods on DON reduction in baking flour. Normal cleaning involved movement of wheat through a cleaning house (60 bushels/h) with multiple screens and suction to aspirate the screenings. Other cleaning treatments included running the wheat through the cleaning process twice (double), increasing the suction (high aspiration), and running the wheat through a wheat washer following the normal cleaning so that the wheat was thoroughly wetted (water washer). Five naturally contaminated (1.85 ± 1.85 mg/kg) and two uncontaminated lots of commercially grown hard red wheat in Kansas were cleaned by described methods, milled and baked into western type bread, one loaf of bread per lot of wheat and treatment (28 loaves). All samples were analyzed by HPLC in duplicate with separate samples for the crust and the crumb. There was no significant difference between cleaning methods for reduction of DON and none of them were particularly effective with 61 ± 4.5% reduction compared to the uncleaned wheat. There was no apparent difference between the DON concentrations in the crust versus the crumb and an overall reduction in DON levels (-22 ± 15%) with less reduction at higher concentrations. Since there was no experimental replication, the significance of any of the results could not be evaluated. This report does not substantiate the previous report of reduction in crust concentration due to thermal alteration although recovery studies were not reported for the analytical method. This study identifies that millers may not be able to effectively alter current cleaning and milling processes to utilize naturally contaminated grains.

Processing reductions in DON contaminated corn has been investigated for making tortillas. Abbas et al. (1988) used two stored corn samples, one of which had been identified

as causing feed refusal in pigs 13 years previously. Naturally contaminated samples were compared to uncontaminated corn and corn spiked with zearalenone and DON (1 ppm or 5 ppm each) or DON alone at 10 ppm. The corn was processed and tortillas were prepared by a domestic Mexican method which included an aqueous 2% Ca $(OH)_2$ boil and overnight soak. Unprocessed corn, finished tortillas, and soaking water were analyzed by HPLC for DON and 15-ADON with TLC and GC/MS confirmation. Zearalenone concentrations were determined by HPLC with GC/MS confirmation. The two stored corn samples contained all three mycotoxins with zearalenone at 0.23 ± 0.08 and 4.23 ± 0.65 ppm, DON 3.28 ± 3.64 and 12.26 ± 3.01 ppm, and 15-ADON 1.49 ± 1.07 and 9.83 ± 1.62 ppm, respectively. Tortilla processing reduced zearalenone by 59.1% in the more contaminated sample and 100% reduction in the lesser contaminated sample but the reduction did not appear to be due to loss into the soaking water. DON levels in the tortillas was consistently reduced compared to the original corn in all samples (71.9 to 82.4%) although soaking water concentrations was not reported and no statistical analysis was indicated to assess significance of reduction. 15-ADON was reported to be completely removed by the tortilla process. Alkaline conditions seemed to partially degrade these mycotoxins but the only limit of detection for the analytical methods reported was for the HPLC analysis of DON (10 ng) and 15ADON (500 ng). A detection method with lower limit of detection may have characterized the disappearance of 15-ADON better. Other than one report by Greenhalgh et al. (1984) described previously, characterization of degradation products chemically or toxicologically have not been conducted.

As the DON contamination of wheat varieties spread across Canada in the 1980's, concerns arose about the influence of processing on DON contamination in different food products made from this wheat. Nowicki et al. (1988) studied the retention of DON in wheat processed to spaghetti and noodles. Two varieties of wheat naturally contaminated with DON (12.5 and 9.6 mg/kg) were milled and processing into Chinese and Japanese noodles and spaghetti. The pastas were cooked and the cooking water collected and freeze-dried. Analysis was by GC-ECD and GC-MS for all samples. Recovery studies included spiking a control lot of both wheat varieties with DON at 5.0 and 0.5 mg/kg, then processing to semolina and spaghetti. Total recoveries were greater than 90% with cooked spaghetti retaining 50% of the

original DON spike regardless of the spiking concentration. Japanese noodles retained an average of 110.9 ± 6.2% of the DON in the noodle and the cooking water, with even distribution. Chinese noodles exhibited a loss of DON with retention averaging 57.2 ±0.8% in the noodles and the water. This was attributed to addition of a commercial preparation of carbonate and phosphate salts of potassium and sodium that represent 1% of the recipe. This resulted in alkaline conditions that contributed to DON degradation similar to tortilla processing. No pH measurements of the cooking water were reported so this was not confirmed. Spaghetti results were similar to the Japanese noodles with total retention of 92.5 ± 7.7% DON in the pasta and the cooking water with 48.6 ± 3.9% in the noodles alone. Overcooking the noodles increased the leaching of DON into the water by another 6%. This work did not report any experimental replication therefore statistical significant of treatments could not be evaluated. These non-staple food products may have reduced DON levels in the consumed forms but Japanese style noodles may be consumed as soups retaining all of the mycotoxins present in the uncooked product. Further work would be needed to characterize the DON degradation products under the alkaline conditions proposed for the Chinese style noodles.

Another type of food product cereal-based with the potential for DON contamination is extruded snacks. Baking has not proven to be a method of DON decomposition therefore Accerbi et al. (1999) investigated extrusion of DON contaminated wheat following treatment with aqueous sodium bisulfite solutions (0.0, 0.5, 1.5, 2.5 or 5.0% SO_2 equivalent) for 1 hour. The original DON concentration of the soft white wheat was 7.3 mg/kg and analysis of experimental samples was by GC-ECD. Whole wheat was soaked in the treatment solution, drained and dried overnight. Two extrusion protocols, low temperature (max. 140°C) and high temperature (max. 170°C), were followed with milled whole meal and flours from the soaked samples (n = 3 per treatment). DON concentrations were significantly reduced by sodium bisulfite but ≥1.5% sodium bisulfite treatments were not more effective. The authors reported statistical analysis in the methods but no standard deviations were given and limited assessment of significance of results. The low temperature protocol was slightly more effective which was attributed to instability of DON-S adducts as reported by Young et al. (1986b) (see page 20). From a food processing perspective, the addition of sodium sulfite to

flour for baking resulted in unsatisfactory consumables whereas the finished extruded product appeared to have acceptable odor and appearance. This study utilized highly contaminated wheat (7.3 mg/kg) with treatments resulting in DON levels (0.6 mg/kg in 1.5% SO_2, low temp. extrusion) that would be acceptable under current FDA advisory level for food for human consumption.

Numerous other food products with the potential for mycotoxin contamination are processed by retorting, food processing similar to autoclaving. Wolf-Hall et al. (1999) studied the effect of extrusion on corn grits and dog food and the effect of autoclaving on cream-style corn and wet dog food with regard to DON retention. All food samples were spiked; corn grits with crude extract from *Fusarium graminearum* culture to achieve 4 mg DON/kg, purified DON was used to spike commercial dog food powder mix (5.47 mg/kg), locally purchased canned cream-style corn and liver-flavored canned dog food (3 mg DON/kg). The canned products were removed from the original containers, mixed with the spiking solution and refrigerated overnight prior to autoclaving at the conditions consistent with commercial retort pressure and temperature. Analysis was done by ELISA with limit of detection 0.3 mg/kg, three replications of the experiment and two analyses per experiment. An additional set of analysis was conducted with inclusion of α-amylase to the aqueous sample extraction before DON determination by ELISA. The addition of α-amylase was to improve the filterability of the cream-style corn but surprisingly had a significant improvement in the recovery of DON from the processed samples. The recovery samples revealed 71 to 100% recovery without α-amylase but increased to 89 – 120% with the enzyme in the extraction. The sample results were more dramatic with 226% increase in DON recovery from extruded corn grits and all samples showed increased recovery 125 – 141% compared to the processed samples without α-amylase. The only process that showed an influence on DON retention was autoclaving cream-style corn, 12% less DON than the unprocessed control. This study determined that these common food processing techniques do not significantly reduce DON levels in food and the possibility of heat sensitive binding between food carbohydrates and DON. This binding would most likely be released when the food enters the mammalian gastrointestinal tract. DON analysis in heat-treated foods may

underestimate the contamination. Further investigation into this interaction has not been reported thus far.

Since heat processing has not been shown to reduce DON levels, studies have investigated recipe additives to promote DON reduction. Young et al. (1986a) tested chemical (hydrogen peroxide ± sodium hydroxide, sodium hypochlorite, sodium bisulfite, ascorbic acid, ammonium hydroxide, hydrochloric acid, sulfur dioxide gas and ozone) treatment on soft white winter wheat naturally contaminated at 1 ppm. Following the various treatments, wheat was milled and the flour was baked into cookies, cakes, crackers, muffins, and pancakes. Samples of milling fractions and final products were analyzed by GC-ECD. The greatest reduction in DON level (98%) was by the addition of 750 ml 10% aqueous sodium bisulfite per kg grain for 16 hours. After the treated flour was baked into a final product, DON levels increased 3 – 6 fold compared to the unbaked samples with an overall reduction of 31 – 51%. Other treatments resulted in 60% reduction at best. The flour product after prolonged sodium bisulfite treatment was deemed unsuitable for commercial use.

Another group attempted to add ingredients to reduce DON contamination. Boyacioglu et al. (1993) investigated oxidizers (potassium bromate and L-ascorbic acid), reducing agents (sodium bisulfite and L-cysteine) and ammonium phosphate at maximum allowable levels added to bread recipes using a whole wheat flour contaminated with 3.13 ppm DON or a spiked flour (2 ppm ending concentration in bread). Flours and breads were analyzed by GC/EC with confirmation by GC/MS and method recovery reportedly 87%. Baking bread without additives resulted in 7% reduction of DON, while the oxidizing agents had limited reduction (L-ascorbic acid -14.1 to -25.9%) or increased levels (potassium bromate +4.8 to +10.5). Significant reductions were achieved with the other agents (-36.7 to -45.4%) but depending on the starting concentration, may not be adequate to assure safe consumption levels.

Since microorganisms are used to produce some food products, microbial degradation of mycotoxins has been investigated by several groups. Boswald et al. (1995) investigated disappearance of zearalenone and DON in food related yeast incubations. Yeast species were chosen based on importance in food processing or spoilage. Standard liquid cultures were prepared and cell concentrations normalized prior to mycotoxin exposure. Zearalenone and

DON were incubated with the organisms separately and analyzed by HPLC following 24 hour exposure. Zearalenone was extensively metabolized by all the yeast species to which it was exposed whereas DON was unchanged by the four experimental organisms in concentrations 0.0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/L after 8 days incubation. There were no morphologic changes or apparent inhibition of growth of the yeast cells reported. The test organisms were yeasts associated with cocoa fermentation, wine production, and beer spoilage. The investigators used a good concentration range but may have tried a higher range to determine if the organisms' behavior would have changed. Differences observed between the two mycotoxins tested was most likely associated with the chemistry of the compounds since zearalenone is a steroid and the yeast have biochemical mechanisms for related compounds whereas they do not have mechanisms to degrade DON or it was unable to cross the cell wall due to its hydrophilicity. From this study and lack of degradation by bread fermentation yeasts discussed previously, yeasts are not promising detoxification organisms.

Current food processing techniques did not significantly contribute to the remediation of DON in human or animal foods. Grain milling did reduce contamination by partitioning DON into the fractions, highest levels in the bran, but reduction effectiveness depended upon the penetration of the fungi into the flesh of the kennel (Seitz et al., 1986). Baking (Scott et al., 1983), extrusion (Accerbi et al., 1999; Wolf-Hall et al., 1999), or boiling (Nowicki et al., 1988) reduced the original toxin level up to 50%. Alkaline processes had the greatest reductive effects (Abbas et al., 1988). Addition of approved food additives produced a product that appeared to have reduced toxin levels but further processing by heat or digestive enzymes reversed this effect (Boyacioglu et al., 1993; Young et al., 1986b, Wolf-Hall et al., 1999). Some additives produced a product that was unacceptable for continued processing or consumption (Young et al., 1986b). Thus far, food processing technology is not available to contribute to mycotoxin reduction in the food supply chain.

Detoxification Strategies

Limited methods for utilizing DON contaminated grains for animal feeds have been developed let alone tested for effectiveness. Strategies have included studies with pH and temperature, feed additives, abrasive feed grain treatment, and microbial transformations.

Wolf and Bullerman (1998) performed a controlled study on effects of pH, time and temperature on the disappearance of DON from a buffer solution. Purified DON, 2 μg/ml, was added to citrate-phosphate-borate solution buffered to pH 4.0, 7.0 or 10.0. Three temperatures (100, 120, and 170° C) and treatment times (15, 30 and 60 minutes) were investigated with repetition in triplicate. Samples were analyzed in duplicate by a commercial ELISA kit (limit of detection 0.3 μg DON/ g). All treatments at 170° C had significant disappearance of DON compared to positive controls. Samples with pH 10.0 had significant reduction at all temperatures, disappearance occurring quicker at higher temperature. There are some concerns about this study because the ELISA kit was designed for water extraction of grain at neutral pH and it was not stated if pH adjustment was made prior to sample analysis. Further, treatment times were part of the study design but the samples were heated in oil for these times then held in refrigeration for 24 hours prior to analysis thus continued DON degradation could have occurred that was attributed to the specific treatments. Each treatment was reported to have had at least six analyses but limited statistics were given. Alkaline treatments have been shown to reduce DON levels in grain and this study attempted to characterize the most effective combination of pH, temperature and treatment time but due to some experimental oversight, limited additional information was identified.

Young (1986a) studied food additives as a strategy for decontamination with an extensive investigation of transformation products following treatment of culture derived DON and 3-ADON reacted with sodium bisulfite. DON disappearance was followed over time as well as the reappearance following hydrolysis with various pH solutions, samples analyzed by HPLC/UV directly. Spectral analysis by ^{1}H and ^{13}C NMR revealed the addition of sulfonate side chain to C-10 (figure 2.4) and three alkaline degradation products common to DON-related compounds that occur after extensive reaction times. Although no statistical analysis was reported for this study, the line of reasoning followed seemed sound and provided strong evidence to explain the previous work in baked products.

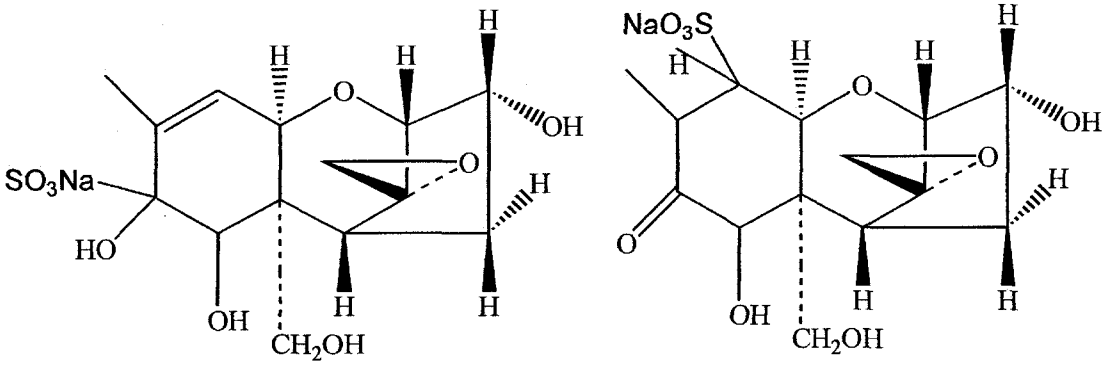


Figure 2.4 Proposed chemical structures for deoxynivalenol sulfonate (DON-S)

Investigation of the detoxification potential of sodium bisulfite treatment was continued by treating experimentally DON contaminated corn with subsequent pig feeding (Young et al., 1987). Acute toxicity tests used nutritionally complete grower diet as four treatments: uncontaminated negative control, negative control treated with sodium bisulfite (0.3% of diet), DON-contaminated corn in diet (7.21 mg/kg), and DON-contaminated corn treated with sodium bisulfite, 0.79 mg DON/kg diet after treatment. An additional acute toxicity test gavaged pigs with purified DON and purified DON-S adduct at effective dose shown to cause emesis in 66% of pigs tested (ED_{66}, 0.096 mg/kg body weight) and 98% of pigs tested (ED_{98}, 0.160 mg/kg body weight) (Prelusky, 1986 unpublished results). Four of six pigs gavaged with the ED_{66} of DON vomited within 3 hours whereas the six pigs treated with the molar equivalent of the DON-S did not show any signs of toxicity. The higher dose caused severe emesis within 20 minutes in all five pigs given the DON but no toxicity signs in three pigs given DON-S. The dietary inclusion of the sodium bisulfite treated DON-contaminated corn resulted in similar performance over 7 day feeding as the negative control diet with or without the sodium bisulfite treatment (n = five pigs per group). Those fed the untreated DON-contaminated diet had significantly reduced feed consumption (870 ± 150 g/pig per day) compared to the other three diets (1500 ± 120 g/pig per day) over the seven day feeding period and significant weight loss (-420 ± 160 g/pig per day) in the first three days of the study where the other three groups had steady weight gain (500 ± 100 g/pig per

day). This study had an appropriate number of animals per treatment to show the toxic endpoints. Since vomiting in pigs was the original toxic effect assigned to DON exposure, this was a specific and sensitive endpoint to use to evaluate the detoxification potential of this grain treatment. It was stated that sodium bisulfite can be fed safely to pigs at double the study level but the practicality of treating contaminated grain in this manner was not discussed. Any further development of this method has not been reported to my knowledge.

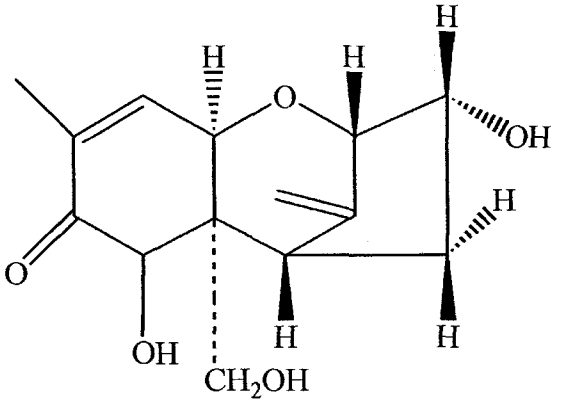


Figure 2.5 Chemical Structure of de-epoxide deoxynivalenol (DOM-1)

Work has been conducted to attempt to detoxify DON by microbial transformation. From early toxicity studies, it was determined that a de-epoxide of DON was produced by cattle (Cote et al., 1986a) and these animals exhibited related reduced toxicity of dietary DON. Cote et al. (1986b) purified and characterized the de-epoxide (DOM-1, figure 2.5) from an incubation of semi-purified DON (300 mg) with rumen fluid supplemented culture media (700 ml) which was 75 – 90% efficient but laborious in purification steps. The experiment was more efficient when DON-contaminated ground corn (15 g of 1300 ppm) was added directly to fermentation media (225 ml). After three days fermentation, no DON was detected by GC/ECD and DOM-1 was purified readily. The purpose of this study was mass production of the de-epoxide metabolite and it led to further studies to investigate the efficiency of microbial decontamination strategies.

Initial studies tried to find suitable sources of microbes capable of transforming DON. He et al. (1992) investigated anaerobic incubation with chicken and swine large intestine contents and aerobic incubations with soil, from a field where *F. graminearum* inoculation in corn had been conducted, on the transformation of purified DON or field-inoculated

contaminated corn (450 ppm DON). Efficiency of conversion under various time and temperature variables was determined by HPLC/UV detection of remaining DON. The chicken intestinal contents had the greatest transformation resulting in 1.8 (± 0.9) % DON remaining after 96 hours incubation. Swine intestinal inoculations and soil were not effective transformers with 99.0 (± 3.9 se) and 91.6 (± 20.2 se) % remaining after 96 hours. Identification of specific bacterial isolates able to transform DON, 20 isolates from chicken gut contents and 5 from soil, was unsuccessful. Although replication of each treatment appeared adequate, significance of findings was poorly defined. They performed the incubations with whole and ground contaminated corn but did not report the condition of the sample following the fermentation, i.e. utility of the end product. Dietary history, age or number of animals from which intestinal contents were collected was not indicated which could affect the microbial profile.

The utility of treating highly contaminated corn with chicken intestinal microbes was demonstrated by He et al. (1993). Grower pigs were fed the following diets for 5 days: standard corn-soybean grower formula, DON-contaminated (4.8 mg/kg) diet made from ground highly contaminated (450 ppm) moldy corn (1.7% of clean corn), DON-contaminated corn treated by incubating with chicken intestinal contents and liquid media (2.1 mg DON/kg diet), diet with clean corn treated with chicken intestinal contents, and DON-contaminated corn added to the diet resulting in the same DON level as the biologically treated corn diet (2.2 mg DON/kg diet). Diets were analyzed for macronutrients and found to be equivalent and adequate. Animals were housed individually and fed *ad libitum*, 3 males and 3 females per treatment diet. Daily feed intake weight gain was significantly lower for the 4.8 ppm DON diet compared to the other four diets. Significantly lower daily gain also occurred in the biologically treated DON-contaminated (2.1 ppm) and the DON contaminated diet (2.2 ppm) compared to the negative control pigs. There were no statistical differences between sexes. Feed intake and weight gain was not different between groups when they were followed for an additional 5 days after experimental diet withdrawal. Treatment of feed with chicken microbes could partially decontaminate (50%) corn although the starting concentration was high. They stated lack of toxicity for DOM-1, concentration assumed to be the difference between the original 4.8 mg/kg and the ending 2.1 mg/kg DON concentration in the diet,

since observed reduced weight gain was similar for both 2 ppm diets. Biologically treated diet was not autoclaved prior to diet addition but did not seem to exhibit evidence of toxicity at the level fed. Appropriate control diets and replication were included to address the objectives. Reduced toxicity as result of the treatment occurred but large scale feasibility was not addressed. Concerns about large amounts of liquid media and incubation at 37°C would limit the utility of this technique.

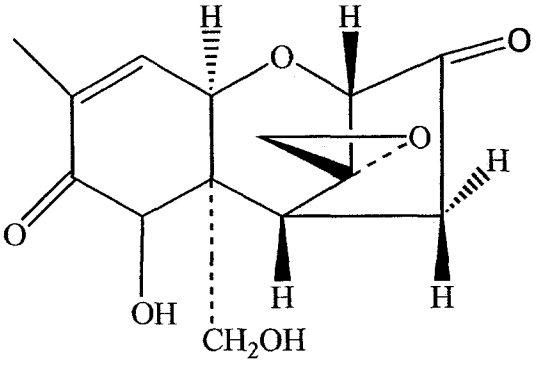


Figure 2.6 Chemical structure of 3-keto-deoxynivalenol (3-ketoDON)

Work to isolate DON transforming microbes continued with Shima et al. (1997) who isolated a soil bacterium which produced a novel DON metabolite. Soil samples were suspended in distilled water and the supernatants were added to enrichment media and incubated for 25 days. The culture was quantitatively added to fresh media supplemented with DON (200 µg/ml) and incubated for an additional 2 to 8 days. Disappearance of DON was monitored by asceptic sampling and HPLC analysis. Soil bacterium from cultures shown to cause DON disappearance were enriched and isolated prior to attempting taxonomic classification with 16S ribosomal DNA analysis. One strain was found (E3-39) and it transformed DON alone in media. The metabolite was identified as 3-keto-4-deoxynivalenol (3-keto-DON, figure 2.6) TLC purification and ^{1}H and ^{13}C NMR characterization. *In vitro* toxicity studies with murine splenocytes revealed significantly reduced toxicity of 3-keto-DON in the tritiated thymidine incorporation assay at 1.0 µg/ml media (about 75% of control), where the parallel DON incubations exhibited about 20% of control at 250 ng DON/ml. They reported triplicate replication but did not provide statistical evidence of this. The side chain on C-3 may be an integral component of toxicity of 8-ketotrichothecenes

where previously the epoxide had been thought to be the main contributor to toxicity. The authors did not indicate the possible application of mass detoxification with this microbe.

A continuous fermentation system was investigated for anaerobic rumen microbial DON degradation and aerobic batch soil microbe incubation (Binder et al., 1998). Rumen fluid from cattle (no specifics on diet or age) was included in a standard media supplemented with 40 ppm DON, for an acclimation period of two weeks, then increased to 180 ppm. The disappearance rate of DON increased from 1.37 mg/g dry matter per day to 2 mg DON/g dry weight with the increase of the concentration. DON and metabolites were monitored by HPLC with purification with TLC and characterization with mass spectroscopy. The main metabolite identified was the de-epoxide and its toxicity was assessed by growth inhibition of *Saccharomyces cerevisiae* bioassay. Organism sensitivity to DON was 50% inhibition of growth at 31 ppm whereas DOM-1 had 10% inhibition of growth at 316 ppm in media. The aerobic soil incubation supplemented with 120 ppm DON had slow transformation with 50% reduction in 48 hours and complete disappearance in 96 hours. Five metabolites appearing by both HPLC and TLC were selectively stained for epoxides, all metabolites retaining this structural characteristic. This would be consistent with Shima et al. (1997) identifying 3-keto-DON as a soil bacterium DON metabolite. Continuous fermentation systems were a possible detoxification technique but utilization of a liquid substrate would require additional DON disposal steps. Industrial application of this type of process does not seem efficient or economically feasible to obtain a functional end product.

A more recent attempt to find a practical microbial detoxification method was proposed by Volkl et al. (2004). They screened 906 mixed and pure cultures from soil and cereal ears, 236 pure cultures from insects, 72 isolates from a water pond, and 70 *Pseudomonas* spp. from an institutional collection. The only culture found to have any DON transforming ability was a mixed culture from spontaneous infected minimum medium with glucose and protein (MMGP). This culture was specified at D107 and maintained its ability to transform DON for at least 6 months. The main DON metabolite was produced over 5 days and resulted in complete disappearance of DON assessed by TLC. The metabolite was identified as 3-keto-DON by mass spectroscopy, ^{1}H and ^{13}C NMR. A specific isolate could not be identified from the mixed culture and the transformation required the presence of

cells. This work was conducted on a microscale and would need considerable effort to scale-up. The authors proposed isolation and investigation of the microbial enzyme(s) responsible since finding a detoxification species was difficult. From the number of species screened in this study, without a specific organism identified, it was suspected that this was a different organism from E3-39. Additionally, the substrate profile was slightly different, D107 being able to transform DON, fusarenon-X, 3ADON, and 15ADON but E3-39 could not transform fusarenon-X. It was further proposed that due to the rarity of the DON transforming ability of these organisms, these substrates are utilized by chance not as primary substrates. The likelihood of this line of research producing a feasible method for remediation of DON contaminated food or feed seems remote.

There have been a few products introduced to the animal feed industry claiming to remediate mycotoxin contamination by adsorption and/or degradation. Doll et al. (2004) developed an *in vitro* model to evaluate the efficacy of these products already available and other possible candidates. The model was a buffer system that mimicked the pH, temperature, and transit time of the pig gastrointestinal tract. Substances evaluated included six commercially available adsorbers (some with unspecified enzymatic activity), one proposed commercial product and three laboratory substances. Detoxification was assessed as removal of DON from the buffer supernatant as analyzed by HPLC. The starting concentration represented a meal containing 10 mg DON/kg diet with 25% dry matter. The only substance that significantly reduced DON concentration in the buffer was activated carbon (67 ± 6% reduction compared to spiked, untreated control). The authors did replicate this experiment but did not provide recovery study data. The comparability between the *in vitro* model and manufacturers' instructions of use was not discussed. There may be other factors involved thus the ultimate test would be *in vivo* assessments.

Avantaggiato et al. (2004) attempted to assess the potential of activated carbon as an intestinal adsorbent of DON and NIV. They used an *in vitro* model that simulated the gastrointestinal tract of the pig and analyzed the mycotoxin distribution through the compartments representing stomach, duodenum, jejunum, and ileum. A feed sample was prepared that contained 2.8 ± 0.3 mg DON/kg and 3.8 ± 0.4 mg NIV/kg diet. Activated carbon was added at 0, 0.5%, 1.0%, and 2.0% of diet. Each six hour experiment was

duplicated and dialysate was collected from each compartment with mycotoxin analysis by GC/ECD. Although the carbon inclusion significantly reduced the simulated adsorption of DON and NIV compared to the negative control, the range was only 29 – 45% for DON and 23 – 41% for NIV. The most efficient reduction was with the 0.5% inclusion level which is the recommended dose and was not likely to interfere with nutritional components of the ration. Since only one dietary DON level was studied, a dose response would be helpful. The concentration used was adequate to cause signs of toxicity in pigs although the adsorption observed in this model may not be adequate to substantially reduce toxicity.

Another approach to reducing the DON contamination of animal feeds was partial processing to remove the toxin. House et al. (2003) investigated the effect of pearling (abrasion to remove hull) on remediation of DON contaminated barley for pig feed. The laboratory scale process was conducted in triplicate on three barley lots of variable DON contamination (4.8 ± 0.5, 9.8 ± 1.3, and 21.1 ± 2.1 mg/kg). The only variable tested was the time of pearling, 0 to 120 seconds in 15 s increments. DON was analyzed in the pearled barley by commercial enzyme immunoassay. The most efficient treatment was 15 s pearling which reduced the DON content by 66 ± 0.7% across samples with a concurrent barley mass reduction of 15 ± 0.2%. Grain nutrient analysis after treatment revealed an increase in digestive energy and reduced non-digestible fiber. The authors provided adequate statistical analysis and experimental replication to support their findings. This work was conducted on a small laboratory scale but should be considered for scale-up for further development. For the most contaminated sample a longer pearling time was required, with increased loss of grain mass, which could be evaluated by a cost-benefit analysis.

Detoxification of DON contaminated food or feed sources have not had much success to date although some processes showed promise. Alkaline treatments caused DON degradation (Wolf and Bullerman, 1998) and treatments with sodium bisulfite produced reversible DON-S adducts under alkaline conditions (Young, 1986a) but seemed to reduce toxicity *in vivo* (Young et al., 1987). Microbial transformations produced two metabolites depending on the microbe. Chicken (He et al., 1992) and cattle microbes (Cote et al., 1986b, Binder et al., 1998) have transformed DON into a de-epoxide (DOM-1) had reduced toxicity in a yeast bioassay (Binder et al., 1998) and in a swine feeding trial (He et al., 1993). A 3-

keto-DON metabolite was isolated from two different sources; a soil isolate (Shima et al., 1997) and a mixed spontaneous culture (Volkl et al., 2004). This metabolite had reduced toxicity compared to DON in a mouse splenocyte bioassay (Shima et al., 1997). Feed additives intended for animal feeds have been introduced commercially but were not proven to be effective by Doll et al. (2004). Removing DON from feed barley by an abrasive pearling process had significant toxin reduction but will need scale-up to determine commercial feasibility (House et al., 2003).

Much effort has been expended looking for methods to remediate DON contamination in food and feed with little success. Continued vigilance is necessary to screen potentially contaminated grains and properly disposal of lots with DON levels higher than allowed for specific purposes. Concerns occur as to the frequency of screening and the potential for contaminated foods on the market.

Methods of Detection

Since initial identification of DON in grain samples, efforts continued to find the most sensitive, accurate and reliable analytical methods for foods and biological samples. Bioassays have also been employed to screen for biological activity of DON, process a large number of samples, and assess relative toxicity of detoxification strategies and DON metabolites. The methods to be reviewed here are the most current with an emphasis on high performance liquid chromatography (HPLC) and additional details on sample preparation, biological sample analysis, bioassays and innovative techniques.

Analytical methods

Two official Association of Analytic Chemists (AOAC) methods exist for DON quantification, thin layer chromatography (TLC) and gas chromatography (GC) (Scott, 1995). Trichothecenes and other mycotoxins were analyzed by a variety of analytic methods as reported in table 2.2 below.

Table 2.2. Analytical Methods for Deoxynivalenol Detection

Method	*Sample*	*Detection limits*	*Special Considerations*	*References*
Thin-layer Chromatography (TLC)	grain	300 ng/g	-hazardous waste production -time consuming	Scott (1995)
Gas chromatography (GC)	grain		-requires derivatization	Scott (1995)
High performance liquid chromatography-UV detection (220 nm) (HPLC-UV)	wheat flour, bran, corn, rice	20 ng/g; determined by lowest spiking samples	-clean up of extracts required -commercial immunoaffinity and charcoal-alumina columns available -derivatization not required -collaborative study reveals wide range repeatability, recovery and reproducibility between labs -multiple trichothecenes detected	Trucksess et al. (1996) Trucksess et al. (1998) Mateo et al. (2002)
High performance liquid chromatography – mass spectroscopy (HPLC-MS)	Corn grain, urine	25 ng/ml DON and DOM-1 in urine	-multiple mycotoxins and metabolites quantified -sample extraction and clean-up similar to HPLC-UV -dexamethasone internal standard	Razzazi-Fazeli et al. (2003)
HPLC-UV	urine, blood, bile, feces	10 ng/ml (blood, urine); 20 ng/ml (bile, feces)	-preparative HPLC recommended for fecal samples -immunoaffinity column clean-up limits sample loading -de-epoxide metabolite detected	Janes and Schuster (2001) Valenta et al. (2003)
Enzyme-linked immunosorbant assay (ELISA)	wheat, corn	0.5 mg/kg dilution required for > 5 mg DON/kg chicken egg yolk antibodies LOQ 0.16 mg/kg	-conjugation to hapten required -cross-reactivity with 3ADON, 15ADON – reduced with chicken egg yolk antibodies -aqueous extraction limits recovery -rapid screening of large number of samples but samples should be batched -poor correlation to TLC	Sinha et al. (1995) Trucksess et al. (1995) Wolf-Hall and Bullerman (1996) Schneider et al. (2000)

Table 2.2. (continued)

Fluorometry	wheat, bran, corn, barley, malted barley	range of quantification 0.5 – 50 mg/kg without dilution	-fluorescent derivatization must be specific for DON to minimize cross-reactivity -sample prep same as HPLC-UV -good correlation with HPLC-UV	Malone et al. (1998)
Fluorescence polarization immunoassay	wheat, corn	not indicated	-requires linking of fluorescent tracer and specific antibody -specialized instrumentation -cross reactive with 3ADON -matrix effects with corn samples -time critical preparation steps -aqueous extraction reduces recovery from corn	Maragos and Plattner (2002)
Electronic nose (volatile compound mapper)	barley		-no sample preparation required -prolonged instrument clearing between samples -good GC-MS correlation for ochratoxin but not DON	Olsson et al. (2002)
Molecularly imprinted polymers (MIP)	purified DON, ZEA		-reusable stationary phase -cross-reactive with nivalenol -alternative to immunoaffinity methods -does not require hapten -not commercially available	Weiss et al. (2003)

HPLC methods, although peer-reviewed (Trucksess et al., 1998), have not yet been accepted as official AOAC methods. Although the variability among HPLC methods was high, there are no reports of internal standard inclusion. Other innovative methods are being developed and show promise of increased specificity and sensitivity. Development of MIP (Weiss et al., 2003) was interesting in that once the polymer was formed the matrix could be reused repeatedly by solvent washing, compared to immunoaffinity columns (which cannot be reused).

Comparison of levels of mycotoxins found in foods assist organizations such as the World Health Organization (WHO) conduct risk assessments concerning human exposures. Josephs et al. (2001) conducted an interlaboratory study to evaluate the methods used for mycotoxin detection worldwide. Blank, naturally contaminated (479 ± 48 and 975 ± 98 ng

DON/g) and spiked (467 ± 47 ng DON/g) wheat samples were sent to 18 laboratories in 12 countries for analysis by the methods employed in the respective lab. The labs were instructed to conduct two analyses on each sample at least one week apart (duplicates within day) and they were to use in-house control solutions. Methods employed by the test labs were HPLC-UV (6), GC with electron capture detection (5), ELISA (4), GC- mass spectroscopy (2) and HPLC – fluorescence detection (1). Extraction and clean-up methods also varied with acetonitrile/water most common (15 labs) and commercial Myco-Sep clean-up columns (9). The overall mean for the spiked wheat sample was 488 ± 187 ng/g and was not statistically different from the target DON concentration. Two outlier values (significantly higher than expected means) occurred in both wheat and corn naturally contaminated samples that were removed from the data sets. Although GC and HPLC methods tended to be within expected range, ELISA consistently overestimated contamination levels which may be associated with reported cross-reactivity with 3-ADON. As a measurement of accuracy of analysis, coefficients of variability (CV) was calculated and found to range between 32 and 41% for between-laboratory reported DON concentration in naturally contaminated samples. The authors indicated one explanation for poor accuracy was lack of distribution of a common control solution. Although an official AOAC GC method has been validated, variability in sample analysis may still occur due to differences in sample preparation, technicians handling samples, different matrixes effects. As was discussed earlier, regulations or guidelines for DON contamination in foods are varied worldwide, with some types of samples at zero tolerance (FAO, 2004). The analytical method chosen for these samples must not only have the specificity and accuracy but comparability to other methods so that mycotoxin levels are universally acceptable.

Bioassays

Although analytical methods provided information on the absolute concentration of mycotoxin(s) within the sample, bioassays were developed to compare the dose-responsive biological activities of the compound(s). Bioassays for trichothecenes utilized eukaryotic cells or cell lines as these were sensitive, although widely variable concerning specific cell used and biological characteristic assayed. Below is a table of bioassays developed for DON screening and specific important aspects of these methods.

Table 2.3. Bioassay Methods for DON Screening

Bioassay Cell Type(s)	*Assay Type*	*Sample & Limit of Detection*	*Special Considerations*	*References*
K-562[1] cell line	MTT[2]	purified DON in media 0.4 µg/ml	-range of linearity 0.1 to 25 µg/ml -MTT assay requires careful dissolution of formazan product with high conc. organic solvent -cells nonadherent and easy to maintain in culture	Reubel et al. (1987)
BALB/c mouse splenic lymphocytes stimulated with PHA mitogen	Inhibition of proliferation by [^{3}H]thymidine incorporation	purified DON; contaminated corn extracts	-solvent extracts dried, redissolved DMSO -IC_{50} [3] (purified DON) 115 ± 23 ng/ml -matrix inhibition not explained by toxins present in extract -up to 4 mg/kg did not affect cell growth curves	Porcher et al. (1987)
K-562 MIN-GL1[4] Human peripheral blood lymphocytes	MTT	purified mycotoxins	-CD_{50}[5] 300 ng/ml (K-562) 400 ng/ml (MIN-GL1) 430 ng/ml (lymphocytes) -15-ADON equally toxic to K-562 and lymphocytes - 3-ADON 5 times less toxic than DON	Visconti et al. (1991)
BHK-21 cell line[6]	MTT MTS[7]	purified DON	-CD_{50} DON 112 ng/ml (MTT) 141 ng/ml (MTS) -MTT result can only be measured once -culture can continue to metabolize MTS after addition	Rotter et al. (1993)
3T3 cell line (Swiss mouse fibroblasts)	BrdU[8] MTT LDH[9]	purified mycotoxins	-BrdU most sensitive assay (IC_{50} = 263 ± 18 ng DON/ ml media -cytotoxicity not determined for MTT or LDH due to insensitivity of cells -adherent cells requiring extra culture preparations -cells only incubated with mycotoxins for 24 hours compared to most other assay 48 hours	Widestrand et al. (1999)

Table 2.3. (continued)

3T3 cell line	BrdU	wheat, wheat bran, oats, barley extracts	-significant matrix effects from oats -cells more sensitive to purified toxin than wheat spiked with DON -IC_{50} (spiked wheat) 1.216 ± 0.094 mg/kg flour -highest conc. extract tested was 400 mg cereal equivalent/ml media -cereal samples prepared as for HPLC analysis except solvent evaporated and residue redissolved in media	Widestrand et al. (2003)
K-562	Trypan blue dye exclusion MTT BrdU	purified DON	-cell viability not affected (Trypan blue) - IC_{50} 651.2 ± 8.9 ng/ml (MTT) 473.2 ± 148.0 (BrdU)	Minervini et al. (2004)
3T3	BrdU	DON, de-epoxide DON (DOM-1) 15-ADON, 3-ADON	-production of DOM-1 from incubation of DON with pig feces and purification by preparative HPLC column (GC-ECD confirmation) - IC_{50} (DON) 444 ± 101 -15-ADON equally toxic as DON -3-ADON 9 X less toxic than DON -DOM-1 52 times less toxic	Eriksen et al. (2004)

[1]K-562 – Human erythroleukemia cell line, able to differentiate into cells of progenitors of erythrocytic, granulocytic, and monocytic cells (Lozzio et al., 1981).

[2]MTT assay- Colorimetric dye cleavage dependent on mitochondrial activity to reduce yellow substrate [(4,5 – dimethylthiazol – 2 – yl) – 2,5 – diphenyltetrazolium bromide] into blue formazan product.

[3]IC_{50} – median inhibitory concentration for proliferation

[4]MIN-GL1 – Human Epstein-Barr virus transformed lymphoid B-cell line

[5]CD_{50} – median cytotoxic dose

[6]BHK-21 – Baby hamster kidney cell line

[7]MTS – Colorimetric dye cleavage similar to MTT except yellow substrate [5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazol-2-yl)-3-(4-sulfophenyl) tetrazolium] is metabolized to a water soluble formazan.

[8]BrdU – Assay for cell proliferation based on detection by immunoassay of DNA incorporation of 5-bromo-2'-deoxyuridine.

[9]LDH – Lactate dehydrogenase released into the media as an evaluation of plasma membrane damage.

Bioassays can be utilized to compare toxicities of compounds rapidly and without use of experimental animals. Bioassays should be relatively easy to conduct and screen many samples simultaneously. Another characteristic that can be incorporated into bioassays is relevance to the toxin's biological effects. Most bioassays listed in the table above use cell types that have not shown evidence of toxicity *in vivo*, such as fibroblast (Eriksen et al., 2004; Widestrand et al., 2003) or kidney cell lines (Rotter et al., 1993). K-562 cells were

found to have linear dose-responsive inhibitory responses in several bioassays (Minervini et al., 2004; Reubel et al., 1987; Visconti et al., 1991). K-562 cells originated from a patient with chronic myelogenous leukemia (Lozzio and Lozzio, 1979). These cells have a high rate of proliferation (46 – 50 X in 7 day culture) and stay in suspension (Lozzio and Lozzio, 1979). K-562 cells have differentiated into progenitors of granulocytes (immature neutrophils), monocytes, and erythrocytes (red blood cells) (Lozzio et al. 1981). These cells may serve as models for bone marrow progenitor cells to which DON was toxic (Lautraite et al., 1997). When human peripheral lymphocyte cytotoxicity was compared to K-562 cell cytotoxicity following DON exposure in media, the cell line was slightly more sensitive than the primary cells, 300 ng DON/ml and 430 ng DON/ ml respectively (Visconti et al., 1991). Bioassays of this type may directly relate the effects observed on the bench to potential *in vivo* effects. Although the true test would be *in vivo* studies, reduction of laboratory animals and expense of doing these trials can be an important benefit to this work.

Bioavailability and Metabolism

Since the initial studies of toxicity of DON, determination of the fate of this xenobiotic in different species has been limited and variable. Some information has been gleaned from studies with alternate objectives. The initial studies were in rats and ruminants with some information available for mice and pigs more recently. There are only a few studies with human data but thus far no definitive evidence for metabolic fate.

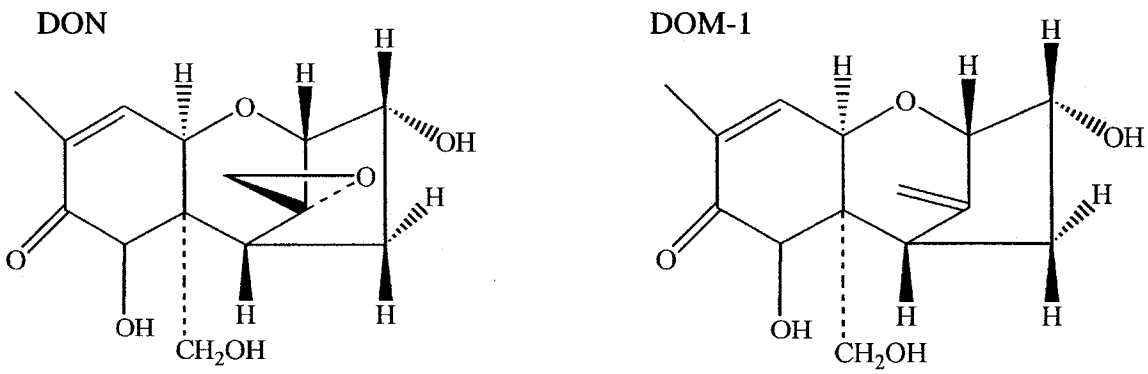


Figure 2.7 Chemical structure of deoxynivalenol (DON) and de-epoxide deoxynivalenol (DOM-1)

The first study to identify a metabolite of DON was from Yoshizama et al. (1983). A bioavailability study was conducted in Wistar male rats, gavaging them with 6 mg DON/kg bw in a single dose. Urine and feces were collected every 12 hours for 96 hours when animals were terminated with collection of plasma and liver. Analysis of DON in samples was by GC/ECD. They reported elimination of DON from the rats within the first 72 hours and peak elimination of DON at 12 hours in both urine and feces. The metabolite (designated DOM-1, figure 2.7) had peak elimination at 36 hours in urine and 48 hours in feces. DON was detected in the liver and the plasma 12 hours after dosing at 63 and 43 ppb respectively and DOM-1 was detected at 24 ppb in the plasma and 16 ppb in the liver. DOM-1 was characterized through GC/MS, negative staining for epoxides on TLC, and a third method that appeared to be related to NMR but was not defined. The evidence presented seemed adequate to report discovery of the DON metabolite but methods were not described fully, number of animals used or proportion of DON eliminated as DOM-1 was not indicated. Studies would be required to elucidate importance of this molecule in the metabolism of DON.

Lake et al. (1987) reported metabolism of radiolabeled DON in four adult male PVG rats. Animals were given a single oral dose of 10 mg DON/kg bw and placed in metabolic cages for collection of urine, feces, and expired air for 96 hours. The dose had been determined from preliminary studies evaluating toxicity in these animals. Analysis of urine, feces and tissues after euthanasia was by acidic and neutral condition HPLC and GC/MS for metabolites. It was found that most of the radioactivity was excreted in the feces (63.6 ± 0.5%) with less in the urine (25.4 ± 1.7%) and only 0.11 ± 0.07% in expired air. The urinary excretion occurred primarily in the first 24 hours after dosing (14.6 ± 2.8% of total dose) but the highest recovery from the feces occurred in the period 24 – 48 hours after dosing (31.0 ± 3.5% of dose). Tissue retention of DON after 96 hours was minimal with the greatest retention in the liver and gastrointestinal tract (0.005% total dose). HPLC analysis of urine revealed five major peaks, 50% radioactivity in two peaks with retention times less than four minutes. Comparing radiochromatograms of neutral and acidic HPLC conditions indicated two non-polar urinary metabolites and a mixture of polar metabolites, most likely DON-conjugates. The fecal metabolite profile appeared similar but with different proportions; polar

conjugates (75%), parent DON (10%) and a non-polar metabolite (15%). Repetitive HPLC purification followed by GC/MS analysis of the non-polar metabolite gave a spectra matching previously identified de-epoxy DON (DOM-1) (Yoshizawa et al., 1983). There appeared to be low interindividual variability of excretion in this study but inadequate information was reported to evaluate metabolite variability as the species of polar conjugates were not proposed.

A contradiction to these findings was reported by Cote et al. (1987) when they studied the glucuronidation of DON and DOM-1 *in vivo* and *in vitro* in rats and pigs. Mycotoxins were incubated with hepatic microsomes from Sprague-Dawley rats and pigs (induced by phenobarbital and 3-methyl-chcholanthrene) to assess mixed function oxidation, NADPH oxidation and microsomal glucuronidation. Residual parent compounds were analyzed by reverse phase HPLC or TLC separation followed by scintillation counting. Three Sprague-Dawley male rats and one female pig were given a single oral dose of DON (2 mg/kg bw for both species) followed by urine and fecal collection for 72 hours for rats and 52 hours for the pig. Excretions were analyzed directly or preincubated with β-glucuronidase, DON or DOM-1 detected by GC/ECD. No metabolism of DON or DOM-1 occurred in any of the microsomal incubations as 98% recovery of the parent compounds was reported for the test systems. No conjugation of DON or DOM-1 occurred in the *in vivo* studies. The pig did not excrete any DOM-1 but an undisclosed amount was detected from the orally dosed rats. This study reported appropriate assay controls indicating the microsomes were functioning but an incubation time of 30 minutes may not have been adequate. Since the *in vivo* study and the rats used for hepatic microsome induction were the same strain but different from the strain in the previous study, strain variability for metabolism of DON should be considered.

Worrell et al. (1989) investigated the role of gut microorganisms in metabolism of DON in rats. Adult male Sprague-Dawley rats were given a single oral dose of 10 mg [^{14}C] - DON/kg bw followed by collection of urine and feces for 72 hours. Additional animals were dosed similarly following microbial depletion with antibiotics (bacitracin sulphate, neomycin sulphate and tetracycline hydrochloride). *In vitro* incubations were conducted using strictly anaerobically collected rat cecal contents with [^{14}C]-DON (35 µg/ml). Sample analysis was HPLC separation and determination of radioactivity in collected fractions measured by

scintillation counter. Urine collected from normal rats had four main radiolabeled peaks, one was parent DON, another was DOM-1, and two were unidentified. The antibiotic treated rats had one of the unidentified peaks and the parent DON but no DOM-1. Fecal radio-elution was similarly affected with 13.3 ± 1.9% radioactivity co-eluting with DOM-1 in the first 24 hours but antibiotic treated rats had only 0.9 ± 0.6%. Incubation of DON with cecal contents, 4 pooled rats, resulted in a steady conversion to DOM-1 with 90% of radioactivity co-eluting with DOM-1 in 24 hours. Individual rat cecal incubations revealed substantial variability of DOM-1 production with 74 ± 24% (29% to 89% range) over 7-hour incubations. The authors reported lack of de-epoxidation if strict aerobic conditions were not maintained during the cecal content collection or incubations. This study was well controlled and provided evidence that gastrointestinal organisms were responsible for de-epoxidation of DON in the rat. Interindividual variability was evident from the *in vitro* incubations after 7 hours but not the *in vivo* which may indicate ability of the gut microbes to maximally biotransform DON during gut transit time.

Absorption and tissue distribution of DON in mice has been investigated in only one published report as part of a larger study. Azcona-Olivera et al. (1995) gavaged mice with [^{3}H]DON at 5 and 25 mg/kg bw with tissues collected at 0.5, 1, 2, 4, 8, and 24 hours after dosing, 3 mice per group. Plasma and tissues were solubilized and mixed with scintillation cocktail for measurement of residual radioactivity. Peak plasma [^{3}H]DON occurred within 30 minutes for both doses and plasma clearance after 24 hours was approximately 94% and 88% of dose in mice given 5 and 25 mg/kg respectively. Toxin elimination appeared to follow two compartment kinetics with rapid initial disappearance ($t_{1/2}$ = 0.36 hr 5 mg/kg, $t_{1/2}$ = 0.56 hr 25 mg/kg) and slower terminal clearance ($t_{1/2}$ = 7.62 hr and 88.9 hr). The 25 mg/kg dose resulted in a secondary DON peak at 4 and 8 hours after dosing suggesting enterohepatic recirculation. No attempt was made to define the metabolite profile. Since terminal clearance was prolonged at the higher dose, toxicity studies at this dose level should consider the longer term effects of single or repeated exposures.

The fate of DON has been investigated in cattle and sheep. Ruminant species were more tolerant to higher dietary contamination with DON which may be explained by differences in metabolism from non-ruminants. Three dairy cows were fed 66 ± 11 mg

DON/kg diet (Cote et al., 1986a) with variability in diet concentration due to daily milk production based feeding. Cattle were fed the contaminated ration twice daily during milking for 5 days. Urine, feces, and milk were collected 5 days prior, during, and three days after feeding the DON contaminated diet. Samples were analyzed by GC/MS for DON and DOM-1. Urine from one cow was preincubated with β-glucuronidase to assess presence of glucuronide metabolites. There were no significant differences between animals for feed consumption or milk production over the feeding period and no adverse effects were reported. Unconjugated DON was not detected in milk but unconjugated DOM-1 was detected 12 hours after DON feeding began, was not detected 24 hours after last feeding with peak concentration of 26 ng DOM-1/ml milk. Unconjugated DON and DOM-1 was detected in urine and feces within 12 hours of start of feeding and continued for up to 3 days after the 5 day trial, with peak excretion occurring after three days on the contaminated ration. Incubation of one cows' urine with β-glucuronidase resulted in 1.6 to 3 fold increase in DON detected and 7 to 16 fold increase in DOM-1 detected. This trial reported consistent excretion, although low concentration, of a DON metabolite into a major human food but it would have been important to know the concentration of conjugated metabolites being excreted with additional studies on the survival of metabolites through milk processing and digestion following human consumption. There was high variability in excretion data due to milk production based DON feeding because milk production was between 19.2 ± 2.1 and 11.7 ± 1.0 kg per day but there appeared to be an accumulation of DON and metabolites since peak excretion occurred 3 -4 days after start of feeding with maximum 18.0 μg/ml unconjugated DOM-1 in urine from one cow. Differences in route of excretion between cows were not discussed but cow #2 had the highest peak urinary excretion while being the median milk producer. Cow #2 was also the lowest fecal excreter with peak DOM-1 excretion 2200 ng/g compared 13,000 ng/g excreted from the highest milk producer. This study provides evidence for the tolerance of cattle to high DON contamination due to their capacity to biotransform DON although the variability between animals could not be explained by the difference in intake alone. The difficulty of a feeding study of this type was not indicated although several data points were omitted most likely due to urine collection problems.

The study of the metabolism of DON in ruminants was more aggressively undertaken by Prelusky et al. (1985) using sheep as a model. Initial work focused on plasma pharmacokinetics using adult castrated male sheep. DON was administered intravenously (i.v.) to sheep at 0.5 mg/kg bw (3 animals) and directly into the rumen through a surgically placed fistula at 5.0 mg/kg bw (4 animals). Blood and rumen fluids were collected for 30 hours following ruminal administration, blood was collected for 10 hours post-dosing for i.v. administration. DON and DOM-1 was detected by GC/ECD with extent of conjugation determined by incubation of samples with β-glucuronidase to determine difference in DON and DOM-1 concentration from unhydrolyzed samples. For i.v. dosing, two-compartment elimination was observed with initial distribution occurring quickly ($t_{1/2\alpha}$ = 12-23 minutes) and the terminal elimination phase occurring over a longer period ($t_{1/2\beta}$ = 67 min mean, 57-78 min range). Peak conjugated DON concentration occurred at 77 min (60-90 min range) and accounted for 15 to 23% of total DON detected in plasma. DOM-1 was detected in plasma, 1.4 to 1.7% of administered dose. Following ruminal dosing, DON was detected in the plasma within 30 minutes and peaked at 583 ng/ml (470-760 ng/ml range) between 4.0 to 5.3 hours post-dosing. DOM-1 was detected in plasma following the rumen dose but only accounted for 1.8 – 2.8% of DON dose given. Glucuronide conjugated DON accounted for an apparent 63-86% of dose. Bioavailability of DON, defined as the ratio of AUC oral dose over the AUC of i.v. dose, was calculated as 7.5% (5.9 – 9.9%). To determine the fate of DON in the rumen, rumen fluid was monitored for DON concentration and a cobalt marker (rate of disappearance indicated outflow from the rumen). DON's rate of disappearance from the rumen was 2-3 times faster than the cobalt marker suggesting substantial microbial metabolism. Hepatic biotransformation of DON to DOM-1 was also suspected since a low concentration of this metabolite occurred in the plasma from i.v. dosed sheep. Low bioavailability and substantial biotransformation of DON in the rumen helped to explain the tolerance of ruminants to higher doses of DON from contaminated feed sources.

Prelusky et al. (1986) followed-up with investigation of DON elimination using adult castrated male sheep which had gall bladder and urinary bladder surgical catheterization prior to oral and i.v. dosing with 5.0 mg/kg and 0.5 mg/kg respectively. Bile and urine was periodically collected for 14 hours in two animals following i.v. dosing and for 36 hours

from two sheep dosed orally. GC/ECD was used to analyze samples following incubation ± β-glucuronidase to determine conjugation of DON and DOM-1. Both routes of administration resulted in excretion of free DON and conjugated DON and DOM-1. No free DOM-1 was detected by either route. Intravenous dosing resulted in 63.2 ± 2.7% total excretion in urine; 24.1 ± 0.7% as parent DON, 21.3 ± 1.8% conjugated DON, and 17.3 ± 0.1% conjugated DOM-1. Biliary excretion was 3.5 ± 0.1% of dose administered. Oral dosing resulted in much lower percent recovery of all DON metabolites; 2.1 ± 0.4% unchanged DON, 3.6 ± 0.3% conjugated DON, 1.2 ± 0.5% conjugated DOM-1, and 0.06 ± 0.02% free DOM-1. Fecal excretion was determined from the orally dosed animals only; 40.0 ± 8.5% as DON and 24.5 ± 6.4% as DOM-1. Total recovery of administered dose was 70 and 63% for oral and i.v. dosing. Urine and bile was incubated with sulfatase (enzyme to deconjugate sulfonide conjugates) with no change in DON or DOM-1 detected. This elaborate study only used two animals per route of administration but helped to confirm the data of Cote et al. (1986a) and support previous findings of Prelusky et al. (1985) but additional gaps of knowledge were revealed. Neither route had complete recovery of dose nor an attempt to postulate the fate of the missing 30%. It was also interesting the high proportion of conjugated DOM-1 excreted from i.v. dosed animals even though the authors did not think enterohepatic circulation contributed much in distribution and metabolism. De-epoxidation had not been identified from any source other than gastrointestinal microorganism although it was suggested in the previous study. They also did not indicate the recovery time following surgical placement of the bladder cannulae but it was indicated that proper placement was confirmed on necropsy. The stress of the surgery and the diet of these animals could have influenced the microorganisms of the rumen resulting in population changes that could influence DON biotransformation.

An *in vitro* study incubating DON (1.25 mg) with rumen microbes in anaerobic culture media (37.5 ml total volume) revealed biotransformation of DON to DOM-1 over 48 hours (Swanson et al., 1987). Samples were removed from the incubations every 12 hours followed by analysis by GC/MS. Only one DON metabolite was detected which matched the spectra of DOM-1. This study provided confirmation of rumen microbial biotransformation of DON to DOM-1 but strict anaerobic conditions may not have been followed resulting in

reduced microbial concentration reported over 48 hours. Worrell et al. (1987) reported the importance of strict anaerobic conditions to achieve complete de-epoxidation from rat cecal contents. The importance of incubation conditions may also explain the difference between *in vitro* results reported here and the high concentrations of DOM-1 excreted from DON fed cattle (Cote et al., 1986a). This study only used one cow's rumen fluid and cows on different rations may have different microbial profiles.

Coppock et al. (1985) investigated the metabolism of DON in female crossbred pigs with i.v. dosing two animals (0.5 mg/kg bw) following implantation of bilateral central venous lines and urinary catheters. Two controls were included in the study and one of the treated pigs was given i.v. saline infusions to study the effect increased urinary output on DON excretion. Blood for DON level determination was collected from catheters 10 times in the first hour, 1.5, 2 hours and hourly for 24 hours after i.v. infusion of DON dissolved in ethanol. Serum clinical chemistries were analyzed from a separate blood sample taken hourly. Urine for DON analysis and urine chemistries were collected every 15 – 30 minutes. Both pigs exhibited acute toxicity following dosing, vomiting commencing within 7 minutes, diarrhea in 1.5 hours, semiconsciousness in 5 – 6 hours with recovery 11 and 13 hours after initial dosing. The animals finally ate 19 hours after dosing and appeared normal at termination 24 hours after dosing. Pharmacokinetics of both pigs fit a one compartment model indicating linear elimination and limited prolonged release from 'storage' sites such as muscle or adipose tissue. Urinary elimination of DON from the saline infused pig was 57.2% of the dose and 28.1% from the other pig and the plasma half-life was 2.08 and 3.65 hours respectively. The only tissue with residual DON was the kidney with 24 ng/g. As an early study in the pharmacokinetics of DON, no fecal or bile data was collected although the authors suggested hepatic clearance or metabolism to account for the large percentage of DON not recovered. No residual tissue DON was found but pigs are fed *ad libitum* therefore levels could accumulate. This study provided some preliminary data but there was a 10 kg difference between the weights of the treated pigs and each pig was treated differently. The dose appeared to be excessive since the animals all but went into shock which most likely affected the elimination in the pig not given i.v. saline.

Friend et al. (1986) conducted a metabolic study as part of chronic feeding trial for market pigs. A diet was formulated from contaminated wheat (5.26 mg/kg diet) and compared to a wheat based standard grower diet. Seven littermate pairs of pigs were assigned to control or test diet and following a week acclimation to diet and experimental conditions, feces and urine was collected for 5 days. Aliquot samples were taken from 24 hour urine collection and mixed pooled individual feces. Analysis for DON and DOM-1 was by GC/ECD after all samples were incubated with β-glucuronidase. The total amount of DON consumed in a 24 hour period was 3.50 mg, range 2.49 to 4.57 mg. DON was excreted in the urine 63% of ingested, 7% in feces, 6% in urine and feces as DOM-1, and 23% unaccounted. The authors reported an apparent 67% absorption of DON due to high urinary excretion of DON and DOM-1. All samples were hydrolyzed prior to analysis so proportion of DON and DOM-1 excreted as glucuronide conjugate was not determined. High variability of DON intake occurred due to acceptability of the toxin contaminated diet. Animal inclusion in this study was not random because dietary acceptance was determined prior to inclusion of the pair, animals must have eaten at least 0.6 kg of DON contaminated diet per day. This may have caused bias in metabolism results since the animals able to tolerate this level may have hepatic detoxification strategies as yet not defined.

To further define the metabolism of DON by the pig, Prelusky et al. (1988) conducted a key study following the fate of [^{14}C] DON by oral and i.v. dosing. Yorkshire castrated male pigs 10-14 weeks of age were anesthetized for gall bladder, urinary bladder, jugular vein, and stomach cannulation. Animals were allowed to recover for 3-4 days. Orally dosed animals were given 0.6 mg [^{14}C] DON/kg bw through the stomach cannula and animals assigned to iv dosing were given 0.30 mg [^{14}C] DON/kg bw directly into the vugular vein. Blood samples from both groups was collected periodically for 24 hours, fecal samples were collected from the metabolic cages hourly and urine and bile were collected continually and sampled for 24 hours. Measurement of radioactivity in samples was by liquid scintillation counting following oxygen combustion. DON and metabolites were separated and detected by GC/MS. Signs of toxicosis (retching, emesis, pallor) occurred in pigs dosed by both routes but was transient and did not result in subject removal. Plasma elimination followed either biphasic or triphasic models. Two pigs exhibited a rapid distribution phase ($t_{½\alpha}$ = 6.57 ±

2.24 min.) and longer terminal phase ($t½\beta$ = 3.59 ± 0.12 hrs). Three pigs had distribution split into a rapid phase ($t½\alpha$ = 5.84 ± 1.03 min) and prolonged phase ($t½\beta$ = 1.61 ± 0.21 hrs) followed by prolonged elimination phase ($t½\gamma$ = 8.52 ± 1.48 hrs). Following oral administration, plasma levels peaked in 15 – 30 minutes and remain unchanged for about 9 hours before slowly declining, half-life of elimination was calculated as $t½\beta$ = 7.14 ± 2.13 hours. Bioavailability based on comparing AUC of oral dose versus iv dose was calculated as 54.9 ± 8.4%. Urinary and biliary excretion following iv dosing was 93.6 ± 9.0% and 3.5 ± 1.1% of the administered dose respectfully. Intragastric administration resulted in fecal excretion of 20.3 ± 5.8%, urinary excretion of 68.2 ± 14.2% and bile containing 2.2 ± 1.1% of administered dose. Only minor metabolism was reported as >95% of DON was excreted as parent compound. Plasma protein binding of DON was investigated *in vitro* with spiked (2- 100 µg DON/ml) blood samples where they found only 9.15 ± 3.9% DON was protein bound and this level of binding was independent of spiking level or incubation time. They also determined DON partitioned equally between plasma and red blood cells *in vitro* regardless of DON concentration. The authors found prolonged elimination of radioactive DON from both iv and intragastric dosed animals, radioactivity being detected in plasma and urine at the end of the sampling time (24 hours) even though the levels were near limit of quantification. The apparent volume of distribution (V_d = 1.13 ± 0.37 l/kg) was greater than the total body water (V_c = 0.17 ± 0.03 l/kg) which could not be explained by plasma protein binding indicating distribution through out tissues. This high quality study had adequate numbers of animals and sampling times to provide well defined toxicokinetic parameters for metabolism of DON in pigs. Increased sensitivity of pigs to DON appears most likely is due to increased bioavailability and lack of metabolism of the toxin compared to ruminants or rodents.

More recently, Danicke et al. (2004) fed DON contaminated (4.2 mg/kg) diet to eleven castrated male pigs for seven days to determine the segmental absorption of DON through the digestive tract and occurrence of DOM-1. The diet formulated was nutritionally complete and was restricted fed (1.1 kg plus twice as much water) twice daily to insure complete ingestion in 10 minutes. The pigs were randomly slaughtered at designated times after the morning meal on the seventh day of the trial, one pig per time (1, 2, 3, 4, 5, 6, 8, 15,

18, and 24 hours) and one pig was slaughtered unfed, designating a zero hour control. Digesta was collected from each of the following segments: stomach, small intestine (three equal sections), cecum, colon, and rectum. Digesta and blood was analyzed by HPLC/UV following immunoaffinity column clean-up. DON was absorbed from the stomach and first segment of the small intestine with peak plasma concentration occurring about 6 hours after feeding. DOM-1 did appear in higher concentrations in distal segments and accounted for up to 80% of the recovered DON from the rectum, although only 10% of DON given was recovered from the large intestine. This study was hard to interpret and lacked power as only one animal was slaughtered per time point. A further complication of data interpretation included diet acclimation thus toxicokinetic data based on one dose may contain carryover from previous feedings. The plasma data was interesting in that peak DON plasma level was much later than reported for previous studies and only reached about 14 ng/ml with return to zero by 15 hours post-feeding. Prior studies had given the DON directly into the stomach or had not determined plasma levels. Diet components may effect absorption of DON from contaminated food or feed in monogastric animals (including humans).

Since pigs are an important food source, Prelusky and Trenholm (1992) investigated potential residual DON in edible tissues from pigs following chronic feeding. Samples of kidney, liver, and backfat were taken from three studies of animals being fed 6.0 to 7.6 mg DON/kg diet from naturally contaminated sources and as purified amendment. Samples were analyzed in duplicates by HPLC. Surprisingly, not all tissue samples were positive for DON with 87% of total 187 samples had <10 ng/g tissue. Average tissue residuals was 6.7 ± 9.6 ng/g for backfat, 5.0 ± 9.2 ng/g for kidney, and 3.0 ± 3.5 ng/g for liver and these were not significantly different from each other. These animals had been fed chronically and had been killed within three hours of their last feeding. This study provided evidence that pigs do not significantly retain DON within these tissues although analysis of muscle tissue may have been helpful since it is the bulk of body weight and is the tissue to be consumed by humans.

Very little is known about metabolism of DON in humans. One piece of evidence for urinary excretion levels was reported by Meky et al. (2003). In the search for a biomarker of DON exposure in humans, urine was collected from 15 subjects in two areas of China. Analysis of urine followed incubation with or without β-glucuronidase then HPLC/UV DON

detection and HPLC/MS confirmation. All 15 human urine samples had DON levels greater than the detection limit of 4 ng/ml. Samples from the high risk area had 37 ng DON/ml (14 – 94 ng/ml range) and the low risk had 12 ng DON/ml (4 – 18 ng/ml range) and these were significantly different from each other. High risk subjects were from an area of China where wheat and corn are staple foods whereas the low risk subjects primarily consumed rice based diets. Following enzymatic hydrolysis, DON levels increased by 1.2 to 2.8 fold. The authors were able to confirm the identity of DON in the urine with standard elution with both HPLC/UV and HPLC/MS. The level of glucuronidation in humans appeared to be variable but the small number of samples did not allow speculation. Since all of the subjects had detectable DON levels in their urine, it would be interesting to increase the power as well as make sure negative controls are included in the analysis.

Eriksen and Pettersson (2003) conducted an *in vitro* incubation study with human feces to assess the potential of de-epoxidation. Ten individuals (five of each sex) donated fresh fecal samples which were aliquoted to four tubes (1 ml feces per tube) of McDougal buffer solution. One tube remained the negative control while two tubes were treated with 10 μg 3-ADON or nivalenol (NIV) each, and positive control of 5 μg of each added to tube after incubation. Anaerobic conditions were maintained and incubation occurred for 48 hours at 37°C. Analysis of samples for DON, 3-ADON, NIV and metabolites was by GC/ECD with comparisons made between samples for the same individual. No de-epoxide metabolites were found for either trichothecene and 3-ADON was efficiently deacetylated to DON (78 ± 30%). The absence of de-epoxidation surprised the authors due to this ability observed in ruminants, rats, and pigs. They reported 90-96% recovery of toxins from the spiked sample after incubation. 3-ADON was studied since this mycotoxin was produced commonly by the *Fusarium* strains in the Netherlands but it would have been beneficial to include incubation with DON since there may have been a delay of conversion of 3-ADON to DON that did not allow time for de-epoxidation. There was also the issue of strict anaerobic conditions that was important for the study by Worrell et al. (1989) who used a sophisticated anaerobic chamber to maintain the oxygen depleted environment and observed significant reduction in de-epoxidation with deviation from this method. The subjects were not controlled for diet prior to sample donation and it was reported they were consuming a normal Swedish diet.

Since this was the first report of this type for humans, more controlled studies will be needed with different types of diets to make any conclusions about the capacity of humans to de-epoxide these compounds.

The most complete bioavailability information was available for pigs and sheep. Through some early sophisticated although low power studies in sheep (Prelusky et al., 1985 and 1986) and cattle (Cote et al. 1986) it was evident that their tolerance to highly DON contaminated feeds was due to rumen microbial de-epoxidation of DON into DOM-1. Ruminants also produced a large proportion of conjugated DON metabolites that are excreted via urine, feces and minorly in milk. Swine do not exhibit the capacity to biotransform with <5% excreted as the glucuronide conjugate (Prelusky et al., 1988) and high systemic bioavailability and de-epoxidation capacity (Danicke et al., 2004) but it occurs in the large intestine after very little DON remains in the digestive tract. Rodents seem to be between these two metabolism patterns with glucuronidation and de-epoxidation capacity confirmed in the rat (Lake et al. 1987, Worrell et al., 1989, Yoshizama et al., 1983) but not yet determined in the mouse. Human data was sorely lacking but it was known that some individuals produce DON-glucuronide conjugates excreted in urine (Meky et al., 2003) while human fecal microorganism may not de-epoxidate DON (Eriksen and Pettersson, 2003). Much more work is needed on DON bioavailability.

Toxicity

Mode of Action

Understanding and predicting *in vivo* toxicity depends on elucidation of the molecular mechanism of toxicity of xenobiotics. Early work made generalized conclusions about the trichothecenes. The ability of *Fusarium* trichothecenes to inhibit protein synthesis in eukaryotic cells was compared by Ueno et al. (1969). Using a rabbit reticulocyte [^{14}C]-leucine incorporation assay, they found dose-response relationships for the trichothecenes with an order of potency of diacetoxyscirpenol (DAS) > fusarenon-X > NIV (IC50 of 0.05, 0.25, and 2.5 μg/ ml respectively). Mechanisms of protein synthesis inhibition by these structurally related compounds were more specifically elucidated by Cundliffe et al. (1974). By monitoring uptake of [^{14}C]-leucine in H-HeLa cells and both [^{3}H]-uridine and [^{14}C]-leucine in yeast spheroblasts from *Saccharomyces cerevisiae,* they found 12,13-

epoxytrichothecenes inhibit protein synthesis but do not primarily inhibit mRNA synthesis. Through cell lysis and sucrose gradient centrifugation, the effect on the polyribosome was compared for trichothecenes alone and in combination with antibiotics with known polyribosome interaction profiles. It was found that T-2 toxin and NIV caused inhibition of initiation of protein synthesis while trichodermin caused inhibition of elongation or termination since the former trichothecenes caused rapid breakdown of the polyribosome while the trichodermin polyribosome profile was not different from untreated controls. They proposed that the molecules covalently bound to the ribosomes, more specifically peptidyl transferase, by opening the epoxide ring and that alterations in the side chain of C-15 determine the specific mode of action. One fault of this study was that all trichothecenes and antibiotics were used at the same concentration (25 μg/ml) which is now known to be a highly toxic concentration to cells sensitive for NIV and T-2 toxin. The cells were only exposed for 20 minutes but repetition of this experiment to determine the threshold of effect would be helpful to better define the proposed mechanism.

A partial answer to this question came from Ueno and Matsumoto (1974) when they determined polyribosome breakup to occur within one minute after exposure to 0.01 μg/ml T-2 toxin of rabbit reticulocytes *in vitro*. All the trichothecenes investigated in this study were protein synthesis initiation inhibitors but they found that fusarenon-X, which has an acetyl sidechain at C-4 whereas DON has an –OH at C-4, binds to and inhibits SH- enzymes such as alcohol dehydrogenase, lactate dehydrogenase, and creatinine phosphokinase. The enzymes were inactivated when fusarenon-X was incubated with the enzyme prior to substrate addition (up to 3 mg/ml) but not after. Enzyme inhibition was studied further by Ueno and Matsumoto (1975) when they investigated ^{3}H-fusarenon-X binding to alcohol dehydrogenase from yeast, lactate dehydrogenase and creatine phosphokinase from rabbit muscle. The labeled mycotoxin inhibited alcohol dehydrogenase activity only if incubated prior to substrate addition, 0.6 mM for 15 minutes resulted in 20% inhibition; activity could not be restored with a thiol group protectant, dithiothreitol. It was demonstrated through assessment of radioactivity in gel filtration fractions that the labeled mycotoxin bound to the SH-enzymes proportional to the number of active SH- sites on the enzyme, four in alcohol

dehydrogenase. Further investigation into the biological significance of this finding has not been reported nor has DON been studied for this effect.

Greater understanding of the molecular mechanism of DON toxicity alluded to by previous work was provided by Wei et al. (1974). This work focused on the mechanism of ribosomal binding in trichodermin inhibition of protein synthesis in human HeLa cells and rabbit reticulocytes. By labeling trichodermin they identified that it bound to reticulocyte ribosomes at a maximum binding ratio of 0.44 molecules per ribosome. Binding occurred under conditions of active protein synthesis in isotonic, hypotonic, and hypertonic solutions and at 37°C and 0°C. Competition studies, with other compounds known to function by interference with protein synthesis similarly, inhibited binding of labeled trichodermin indicating a single binding site for these molecules. The authors indicated that since binding to the ribosome was not 1:1, binding may depend on the ribosomal cycle i.e. the length of the protein being translated or the location of the peptidyl-tRNA. Allosteric and/or conformational factors were considered important for explaining a poor affinity of the 60S isolated ribosomal subunit but strong affinity in the polyribosome as well as the differences in mode of action of closely related trichothecene molecules. Up to this point, DON had not been included in any of these studies, since it had just been isolated. To this day limited work has studied DON in similar experiments and the main mechanism of toxicity, protein synthesis inhibition, has been assumed to be virtually the same for all trichothecenes.

DON specific effects on the polyribosome were reported by Ehrlich and Daigle (1987). They investigated the structurally related natural and synthesized analogs of 8-keto-12,13-epoxytrichothecenes effect on protein synthesis inhibition in Vero (African Green monkey kidney) and MEL (murine erytholeukemia) cells. Dose response of protein synthesis was assessed by tritiated leucine incorporation after 30 minute incubations with the compounds. Concentrations resulting in 50% inhibition of protein synthesis (ID_{50}) was the lowest for DON in Vero cells (0.9 µg/ml) compared to the synthesized analogs but the MEL cells were slightly more sensitive to 3,7-DiDON (–OH at C-4 and C-15 but not C-7) (0.8 µg/ml) compared to DON (-OH at C-3, C-15 and C-7) (1.6 µg/ml). Other structurally related analogs had at least 10 fold greater ID_{50} concentrations. The authors also studied the structure-activity relationship on polyribosome profiles of MEL cells after 10 minutes

exposure to the compounds. T-2 toxin was included since it was known to cause polyribosome breakdown. DON at 10 µg/ ml (67 ± 9%) and 3,7-DiDON at 50 µg/ ml (64 ± 9%) conserved the polyribosome as indicated by similarity to the untreated control (66 ± 7%) for percent radioactivity from tritiated uridine remaining with the ribosomal fraction following fractional centrifugation. T-2 toxin (5 µg/ ml) and the other analogs (>100 µg/ ml) caused polyribosome breakdown resulting in 24 ± 12% retention of radioactivity in the ribosome. From the data presented it was concluded that DON and 3,7-DiDON were protein elongation inhibitors where the other analogs were initiation inhibitors. Structural features that seemed to differentiate these two mechanisms were the substituents at C-3 and C-4. If both sites were occupied by hydroxyls or other bulkier side groups, the compounds inhibited initiation but if either site had hydrogens, elongation inhibition occurred. This only held true for the 8-keto trichothecenes because substituents on the A-ring also contributed to toxicity and potency. From the space-filling models of the molecule, there was evidence of interaction between the constituent at C-4 and the 12,13-epoxide. A hydroxyl or acetyl can effectively shield the epoxide but hydrogens would expose the epoxide which was thought to be a key structural component for toxicity. From the information thus far, it can be concluded that trichothecenes cause intracellular protein synthesis inhibition in eukaryotic cells but the structural diversity can dramatically change the specific mechanism of this effect.

The suggestion of other mechanisms of action was made by Thompson and Wannemacher (1986) when they compared *in vitro* and *in vivo* toxicity for more than a dozen 12,13-epoxytrichothecenes. Protein synthesis inhibition (tritiated thymidine incorporation assay) studies were conducted in the Vero cell line and mitogen-stimulated primary rat splenocytes with exposure to various dilutions of mycotoxins. The IC50 for protein synthesis inhibition for DON was 444 ± 39 ng/ml and 252 ± 24 ng/ml for Vero cell and rat splenocytes respectively. 3-ADON exposure had much less effect; IC50 of 9198 ± 1516 ng/ml and 1503 ± 275 ng/ml in Vero cells and rat splenocytes respectively. Lethality trials were conducted on mice injecting 10 mice per concentration per mycotoxin by either intraperitoneal or subcutaneous routes. DON and 3-ADON lethality was not affected by the injection route nor did they have significantly different LD_{50} (dose causing 50% of animals to die within 72 hours of exposure), 45 mg DON/kg bw (30 – 55 mg/kg 95% confidence limit) and 59 mg

3A-DON/kg bw (51 – 85 mg/kg 95% confidence limit). The authors discussed possible mechanisms to explain large differences observed for *in vitro* protein synthesis inhibition that was not observed *in vivo*. The obvious reason for the difference between DON and 3-ADON would be *in vivo* metabolism (deacetylation) that may not occur in the culture systems. A comparison that did not make sense with the current knowledge was a comparison of NIV to DON. NIV had a six-fold greater LD_{50} by subcutanteous injection but DON had six-fold lower IC_{50} for protein synthesis inhibition in Vero cells. The structural difference between these compounds is that NIV has a –OH at C-4 whereas DON has a –H. The authors suggested that more potent metabolites were produced *in vivo* or secondary effects occurred to explain greater *in vivo* potency of DON but they also proposed investigation into mechanisms of action other than protein synthesis inhibition.

More recently, investigation to elucidate these mechanisms have focused on differential responses of mRNA and translation products following DON exposure of mice. In a recent review by Pestka et al. (2004) the complexity of the molecular effects without strong evidence for specific mechanisms was described. Cell signaling pathways have been shown to be activated by DON, as low as 1 mg/ kg bw, through gene induction and activation of several mitogen-activated protein kinases (MAPKs) but the trigger or mechanism of induction has not been elucidated. Shifrin and Anderson (1999) proposed ribosome binding requirement for DON's inhibition of protein synthesis and activation of cell signaling pathways termed 'ribotoxic stress response' but active protein synthesis was not required for some trichothecenes to activate the stress response, indicating another although not independent mechanism of action. These authors compared related trichothecene structures with their ability to activate c-Jun N-terminal (JNK)/p38 kinases in the human Jurkat (T lymphoid) cell line, but no correlation was found. DON was found to be a strong inducer of apoptosis, 13 fold activation of caspase-3 at 2.96 μg/ml for 3 hrs, and a strong inducer of p38 and JNK kinases, 12 fold induction at 2.96 μg/ml for 3 hrs. Only one dose was tested and cell viability was not assessed which may complicate the interpretation of this study since all mycotoxins were tested at 10 μM and large differences in potency occur across these mycotoxins. The importance of this study was the suggestion that mechanisms other than protein synthesis inhibition of cellular toxicity are possible and that structurally related

trichothecenes may not function by the same mechanism, which had previously seemed to be the dogma.

Many studies have attempted to elucidate the site of initial influence of DON on cell signaling pathways. The most recent evidence links apoptosis due to DON exposure at doses ≥ 100 ng/ ml in media to activation of hematopoetic cell kinase(Hck) and double-stranded RNA-(dsRNA)-activated protein kinase (PKR) with cascade activation of p38, p53, caspase-3 resulting in apoptosis (Pestka et al., 2004). Although these authors summarized data that indicated low dose trichothecene upregulation of cytokine, chemokine and inflammatory genes while high trichothecene doses seemed to produce leukocyte apoptosis and immune suppression, there appear to be large gaps in knowledge of the pathways of action of DON.

Trichothecene mechanism of action was initially identified as protein synthesis inhibition by Ueno et al. (1969) but more specifically ribosomal binding to the peptidyl transferase center (Cundliffe et al., 1974) inhibiting active protein synthesis (Wei et al., 1974). Fusarenon-X, a close structural relative to DON, was found to bind to critical –SH groups inactivating three different enzymes (Ueno and Matsumoto, 1975). DON was specifically classified as a protein elongation inhibitor (Ehrlich and Daigle, 1987). The possibility of DON mechanisms of action other than inhibition of protein synthesis was proposed by Thompson and Wannemacher (1986) due to inconsistencies between *in vivo* and *in vitro* DON exposure. Molecular mechanisms involving alteration of cell signaling pathways and gene expression were shown in response to DON exposure (Shifrin and Anderson, 1999; reviewed by Pestka et al., 2004) although the specific site of action has not been determined. The complexity of the system seems underestimated. Ribosomal binding and transcriptional arrest seem too simplified. Although this was a common mechanism for some of the trichothecenes, investigation into relative binding affinity for specific inherent proteins has not been conducted.

In vivo Toxicity other than Immunological

The key papers reviewed here will emphasize toxic effects contributing knowledge to the original work reported within this dissertation and relevance to human health. The first studies of DON toxicity were conducted in swine because incidences of toxicosis in this species were reported from the field. The most common signs of toxicity were emesis and

feed refusal. To better characterize the dose-response of pigs, Forsyth et al. (1977) conducted trials dosing pigs intraperitoneally (i.p.) to determine the emetic threshold and in the diet to determine the threshold of feed refusal and effect on weight gain. Pigs (45.5 kg, n = 4) fed 7.2 ppm purified DON sprayed on a standard diet, ate 59.1% of the amount the control pigs ate over 4 days. A dose-response was observed for pigs (20 kg, n = 4) fed diets with 0, 3.6, 7.2, or 40 ppm purified DON with significant reduction of total weight gain and feed intake at all doses. Pigs fed 3.36 ppm had reduced weight gain 68.7% of controls and daily feed intake 80.2% of controls, both parameters significantly different from controls ($p < 0.01$). Doses as low as 0.1 mg/kg bw given i.p. to pigs (9.4 kg) caused repeated vomiting within 46 minutes with higher doses causing a more rapid effect. Gavage of DON in water (0.2 mg/ml) did not cause emesis at 0.075 mg/kg bw in pigs (9.3 kg, n = 3) but caused one of six to vomit at 0.1 mg/kg and 3/3 at 0.4 mg/kg bw, although it took about an hour until first vomition, i.p. dosing took about half the time. Besides the evidence for emesis and feed refusal from this study, it was also apparent that other emetic factors may be present in naturally contaminated grains. This was due to the observation of complete feed refusal of a feed mixed with naturally contaminated corn analyzed as 12 ppm whereas pigs fed a diet with 16 ppm purified DON ate 31.4% of controls. Although an additional pig was fed a diet with 3.2 ppm DON and 1 ppm zearalenone (ZEA) with no additional feed refusal effects observed due to the ZEA, the diet was only fed to one pig for one day. The contaminated corn was analyzed by GC and TLC with no T-2 toxin, fusarenone, or 3-ADON detected. The authors did not list any other trichothecenes specifically. The feeding trial was conducted over a short period and it was evident from the data that it took a couple days for the full effect of DON feeding to occur. Doses between 3.2 ppm and 0 DON were not fed so a NOAEL was not determined.

Since emesis, feed refusal, and poor weight gain were observed toxic endpoints of dietary DON exposure, trials were conducted to determine if effects on weight were directly related to reduced feed intake. Pigs (8.4 kg, n = 10) were fed a diet containing 10.5 ppm DON and 1.1 ppm ZEA from experimentally contaminated corn (Lun et al., 1985) that was analyzed by GC and did not contain T-2, HT-2, diacetoxyscirpenol, nivalenol, or fusarenon-X. A third treatment group was fed the same weight of uncontaminated diet as a paired DON-fed pig. Total feeding time was 21 days with feed intake, body weight, and blood

parameters being monitored weekly. Overall the DON fed group ate 45% less feed and only gained 50% of the weight of the full-fed controls although similar to pair-fed controls. DON fed pigs did appear to adapt to the diet as it was stated that initial feed refusal was not as great after the first week. There were significant treatment differences in hematocrit (Hct) (DON-fed pigs reduced, $p < 0.05$), hemoglobin (Hb) (reduced in DON-fed pigs in weeks 2 and 3, $p < 0.05$), and red blood cell (RBC) counts (pair-fed pigs reduced in weeks 1 and 2 but not 3, $p < 0.03$). DON-fed pigs tended to have reduced serum calcium ($p < 0.06$) and serum phosphorus ($p< 0.05$) compared to full-fed controls but pair-fed controls tended to have higher serum Ca and lower serum P than DON-fed animals which was suggested as a possible DON effect on mineral absorption or metabolism. Other blood parameters measured but not affected by treatment included: serum total protein, albumin, urea, cholesterol, creatinine, conjugated bilirubin, albumin to globulin ratio, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, and creatinine phosphatase. The authors concluded that effects other than mineral differences appeared to be feed refusal related as similar effects were observed in DON-fed and pair-fed controls but no mechanisms were proposed. It was stated that no emesis was observed in the DON-fed pigs which contradicts the previously cited work of Forsyth et al. (1977) since 0.5 mg/kg bw would have been the dose based on data presented. Both studies had similar weight pigs and used crossbreds but Lun et al. (1985) used castrated males while sex was not stated in the former study. This apparent difference in toxicity between these studies most likely was assosciated with the dosing strategy as this study fed DON-contaminated diet while the previous study gavaged the dose thus gastrointestinal DON levels should have been higher.

More recently, work on reduced feed intake versus DON toxicity has been examined. Rotter et al. (1995) investigated dietary DON exposure to Yorkshire castrated male pigs (12.0 kg) over 42 days with inclusion of pair-fed controls. DON was incorporated into the diet at 4.0 ppm from a naturally contaminated corn that had been found to be free of other trichothecenes and zearalenone. Feed intake was monitored daily in the DON-fed group and that amount was fed to a pair-fed control pig. Pig weights were monitored weekly (more frequently in initial two weeks) and blood parameters monitored weekly. DON-fed pigs ate 20% less feed. Reduced weight gain observed was due to reduced feed intake since the pair-

fed control pigs did not differ in body weight gain from the DON-fed pigs. Absolute and relative organ weights of thymus, spleen, thyroid, kidneys, liver, and adrenal glands were not significantly different between treatment groups. The brain to body weight ratio was increased in the DON-fed and pair-fed groups due to an 11% reduction in body weight without changes in brain weight (control = 1.17, DON-fed = 1.34, pair-fed = 1.42, SE = 0.05). The stomach mucosa was assessed for gross changes to the dietary treatments with the DON-fed group having more corrugation in the fundic region but no histopathology was conducted to determine the possible mechanism of alteration. There was a transient significant reduction of Hb in DON-fed and pair-fed animals after 3 weeks but the DON-fed group matched controls by the end of 6 weeks. Serum proteins also had transient effects with lower albumin levels in DON-fed and pair-fed during week 1 and 2 and lower total serum protein in weeks 2 and 3 in the DON-fed group compared to both control groups but no differences in protein levels occurred at the end of the study. The authors suggested a real reduction of protein synthesis in the liver during a rapid growth phase and the animals adapted, no mechanism given, to the DON-containing diet during the second half of the trial. Reduced weight gain due to reduced feed intake in food-producing animals can have major economic consequences due to increased time to slaughter weight. DON may have effects other than feed refusal such as *in vivo* protein synthesis inhibition.

Investigations using rodents to further characterize the toxicity of DON have identified some similarities and differences compared to effects observed in swine. Acute toxicity in B6C3F1 female weanling mice with DON and 15-ADON were conducted by Forsell et al. (1987). Both mycotoxins were purified from culture and administered by gavage or i.p. injection first in LD_{50} range finding doses of 10, 100, and 1000 mg/kg bw then the refinement by administering four smaller increment doses. Animals were dosed once and killed after 14 days, if they survived that long. Of those that did receive a lethal dose, animals died within 48 hours regardless of route of exposure or mycotoxin. These toxins had LD_{50} values of 78 mg/kg bw by gavage and 49 mg/kg bw i.p. for DON and 34 mg/kg bw by gavage and 113 mg/kg i.p. for 15-ADON (no statistics given). Differences between routes of exposure were suggested to be due to differences of absorption and metabolism with a possible influence from intestinal microorganisms although limited evidence substantiates

this possibility. Organs from animals in this study were examined histologically with minimum doses estimated to cause tissue effects. Intestine, spleen, thymus, bone marrow, and heart were equally sensitive requiring 100 mg/kg bw orally and 60 mg/kg bw i.p. to cause lesions from DON exposure. Tissues were more sensitive to 15-ADON as 40 mg/kg bw orally caused lesions in the intestine; spleen, thymus and bone marrow required 60 mg/kg bw orally, and the kidney was affected at 100 mg/kg bw. All tissues were equally sensitive at 160 mg/kg i.p. for 15-ADON. The kidney was relatively resistant to DON effects as lesions were only detected at 1000 mg/kg i.p. Liver, brain, lung, and pancreas were not affected by either mycotoxin. Lesions in all tissues were characterized as necrotic particularly in immune organs. Acute toxicity in B6C3F1 mice was found to depend on mycotoxin and route of exposure. Animal use was reduced in this study following a reproducible protocol but values may be different for other strains. More significant may be the finding of similar range of toxicity for 15-ADON compared to DON and possibly more toxic with oral exposures. Since 15-ADON can be produced simultaneous with DON from the same fungal strain, it is important to consider 15-ADON exposure when assessing toxicity of foods or analysis of human food for hazard assessments.

Toxicity of DON may involve production of reactive oxygen species (ROS) as acute toxic effects in rats was prevented by dietary antioxidants (Rizzo et al., 1994). Male Wistar rats were fed semi-purified diets either deficient in antioxidants, or containing supplemental selenium (Se) for 2 weeks. Additionally animals on the supplemented Se diet were given one time oral supplements of more Se with or without vitamins C and E, 16 hours prior to a single oral dose of DON (28 mg/kg bw) or T-2 toxin (3.6 mg/kg bw). Each toxin dietary and supplemental antioxidant treatment combination had an appropriate control group. Rats were decapitated 24 hours after the toxin dose. All of the rats fed either of the basal diets had diarrhea (10/10) with fewer exhibiting hemorrhage around the nose, ruffled fur, or lethargy. Se boosting almost completely suppressed toxic symptoms (only symptom was diarrhea 1/10), and no symptoms were seen when the rats were acutely supplemented with Se and vitamins. Acute toxicity was similarly observed with acute dosing with T-2 toxin although antioxidant treatment did not effectively reduce toxic symptoms and may be associated with giving a dose close to the LD_{50} of 4 mg/kg bw. Lipid peroxidation in the liver [measured by

thiobarbituric acid-reactive substances (TBARS)] was increased with both toxin treatments in rats fed either basal diets ($p < 0.05$) compared to the diet control groups. Supplemental Se prior to DON dosing resulted in normalization but the additional antioxidant vitamins significantly reduced TBARS compared to diet controls ($p < 0.05$), which was also observed in supplemented controls not dosed with DON. All groups dosed with either of the toxins, regardless of diet, had decreased liver glutathione levels but supplementation with Se and vitamins remediated some of this effect. Liver antioxidant enzymes, catalase (hydrogen peroxide reduction) and superoxide dismutase (SOD, superoxide radical protection), were generally consumed under the influence of DON or T-2 toxicity but activity was protected with supplementation of antioxidants. Evidence was presented that supports ROS production from acute exposure to DON or T-2 toxin in rats and the protective effect of supplemental antioxidants Se and vitamins C and E. Appropriate diet controls were included and adequate animal numbers although mortality following T-2 exposure did produce imbalance. Free radical production by trichothecenes has not been adequately investigated and may be linked to signal transduction pathways being investigated.

Sub-acute and chronic feeding studies more closely follow the likely human exposures to dietary DON thus several rodent studies investigating the effects of more prolonged exposures will be reviewed. Morrissey et al. (1985) fed male Sprague-Dawley rats (190 – 210 g) 20 ppm dietary DON (purified from culture) for 90 days, including pair-fed controls ($n = 10$). Although limited effects were observed, the DON-fed group consumed significantly more feed (27.3 ± 1.9 g) compared to control (24.6 ± 2.0 g) and the pair-fed controls (24.9 ± 1.2 g), it seemed odd to observe this difference in pair-fed controls. There was no significant differences in body weight gain but the RBC count was reduced in the DON-fed group (6.4 ± 0.6 $10^6/mm^3$) compared to control (7.1 ± 0.8 X $10^6/mm^3$) and pair-fed rats (7.7 ± 1.2 X $10^6/mm^3$). Organ weights, histopathology, or liver proteins were not affected by treatments. No possible explanation was proposed for the increased feed consumption or the reduced RBC counts of the DON-fed rats. Even though appropriate methods and statistics were reported in this study, the food intake data seems suspect since the pair-fed controls should have had similar intake.

A more extensive study was conducted by Arnold et al. (1986) to compare chronic dietary DON exposure in mice and rats. Weanling male Swiss-Webster mice and Sprague-Dawley rats were fed one of the following diets: commercial rodent chow (118 ppb DON), formulated diet containing 80% uncontaminated wheat (53 ppb DON) or formulated diet containing 83% contaminated wheat (6250 ppb DON). Ten mice per group were killed at 7, 13, 18, 24, 95, 110, 124 days on diet with the remaining mice killed on day 137. Ten rats per group were killed on days 7, 21, 35, 85 and 91 days on diet. Significantly lower rate of gain occurred in both rats and mice fed the DON contaminated diet ($p = 0.001$ mice and rats) and this effect could not be completely explained by decreased feed consumption. Transient effects were observed in mice as initially decreased erythropoesis in the bone marrow and spleen then either no treatment effect (spleen) or slight enhancement sustained from day 13 until the end of the study (bone marrow) for DON-fed (6250 ppb) animals compared to the other two diets. They also found significant differences between the uncontaminated wheat diet and the DON contaminated wheat diets: Hct reduced in mice ($p = 0.003$) and rats ($p = 0.014$), decreased Hb in mice ($p = 0.006$), WBC increased in mice ($p = 0.028$), decreased RBC count ($p = 0.026$) in rats, and mean corpuscular hemoglobin content (MCHC) increased in rats ($p = 0.031$). Only two parameters were significantly different between the commercial lab chow and the uncontaminated wheat diet groups; platelet count ($p = 0.005$) and MCHC ($p = 0.013$) were both increased in rats fed commercial rodent chow. Although it was not specifically stated, it was assumed these differences were observed through out the feeding period. It appeared from this study that RBC homeostasis is affected by 6.25 ppm in both rats and mice and these rats were more sensitive to the effects of dietary DON compared to the previously described study. Even though the same strain of rat was used, the dietary source of DON was different and no statement was made regarding other mycotoxins analyzed in the contaminated wheat.

A dose-response 8-week dietary DON study was conducted in weanling female B6C3F1 mice (Forsell et al., 1986). DON purified from culture was added to semi-purified AIN-76A diet with final concentrations of 0, 0.5, 2.0, 5.0, 10.0, and 25 ppm. Two diets with purified ZEA were included, one at 10.0 ppm ZEA and the other 10.0 ZEA plus 5.0 ppm DON ($n = 8$ per group). Mice were fed for 56 days with twice weekly feed intake and body

weight measurements. Diets of 2.0 ppm DON or more had significantly reduced body weight gain ($p < 0.01$) after 24 days on test and through the remainder of the trial. Only the group fed 25 ppm DON had significantly reduced feed intake ($p < 0.01$). Organ weights were significantly reduced by greater than 5 ppm DON but more specifically kidney affected at ≥ 5 ppm ($p < 0.05$), liver ≥ 10 ppm ($p < 0.01$), and spleen at 25 ppm ($p < 0.01$). ZEA at 10 ppm alone in the diet did not have any significant effect on organ weights but the combination of 10 ppm ZEA and 5 ppm DON resulted in reduced liver weight ($p < 0.05$) and reduced kidney weight ($p < 0.01$). Blood parameters required at least 10 ppm DON to cause significant effects; reduced WBC ($p < 0.01$) and monocyte counts ($p < 0.05$) but 25 ppm was needed to reduce lymphocyte ($p < 0.01$) while increasing neutrophil ($p < 0.05$) counts. The only blood parameter affected by ZEA in the diet was significantly increased RBC numbers ($p < 0.01$). It was interesting, although not mentioned by the authors that the spleen, thymus, and liver weights as well as WBC count appeared to be increased by 0.5 ppm DON. The conclusions by the authors suggest mice fed 25 ppm in this study may have been experiencing an antigenic insult since the neutrophil count was elevated with concurrent reduced total WBCs although limited evidence could substantiate this conclusion. This study provided evidence for the sensitivity of B6C3F1 female mice with a threshold of 2 ppm dietary DON. The concurrent occurrence of ZEA in naturally occurring feeds would be unlikely to contribute much to DON toxicity unless highly contaminated.

Robbana-Barnat et al. (1987) also observed low-dose sensitivity to DON in mice. Weanling male BALB/c mice were fed a commercial diet with purified DON from culture incorporated at concentrations 0, 2.5, 5.0, 10.0, 20.0, 50.0, and 100.0 ppm. The experiment was replicated three times, $n = 4$, for one week feedings. A pair-fed control group was included that received the basal diet in the same amount as the 2.5 ppm group. Significant reduction in feed intake was observed in diets with ≥ 2.5 ppm DON ($p < 0.005$) but significant weight reduction was not observed until ≥ 10 ppm was fed ($p < 0.005$). All the animals in the 100 ppm group died within 4 days, equaling 50 to 65 mg/kg bw total intake, which is less than the LD_{50}, and the authors thought this may indicate increased sensitivity of this mouse strain and/or an accumulation of systemic DON since no information was known about bioavailability. Thymic weight was significantly reduced by 10 ppm ($p < 0.01$), and

histologically revealed marked atrophy of both the cortex and medulla. This study revealed short-term feeding low-dose DON effects in BALB/c mice which agree with previous mouse studies.

The mechanism of feed intake reduction has not been identified but was suspected to involve a DON effect on neurochemical signals, particularly serotonin which functions in regulation of emesis. Fioramonti et al. (1993) used male NMRI mice to assess gastric emptying following oral dosing of 10 – 1000 μg/kg bw purified DON, vehicle not given, followed by a radioactive milk-based meal. Gastrointestinal muscle activity was assessed by surgically implanting electromyography electrodes in adult male Wistar rats, allowing 5 days healing then dosing them with 10, 50 or 100 μg DON/kg bw 10 minutes prior to a milk-based meal. A significant dose-response effect was observed on gastric emptying starting at 50 μg/kg bw ($p < 0.01$) but intestinal propulsion was only significantly reduced by the 1000 μg/kg bw ($p < 0.01$). The effect was mediated through 5-HT_3 specifically as inhibition of gastric emptying at 50 μg/kg bw was significantly reduced by 5-HT_3 receptor antagonist ondansetron [10 μg/kg subcutaneous (s.c.) injection] and equal to controls (no DON) treated with the antagonist alone. Selective antagonists for other serotonin receptors were not effective in reducing the gastric emptying effect of DON. The effect of DON was produced locally in the stomach and not centrally because i.v. injections of DON (5 μg/kg) did not inhibit gastric emptying and dosing with ondansetron (i.v.) did not reverse the orally administered DON effect. This study did not attempt to further define the mechanism such as source of increased serotonin, other than enterochromaffin cells, or specific effect on 5-HT_3 receptors. This mechanism would appear to be a non-species specific effect as pigs exhibit feed refusal and vomiting at low DON levels and humans with suspected DON exposures express feelings of fullness and vomiting (Bhat et al., 1989). Further characterization of this effect has not been published.

The publication that appears to be most frequently cited with regards to chronic exposures in mice and risk assessment was Iverson et al. (1995). B6C3F1 male and female mice were fed commercial certified diets containing 0, 1, 5, or 10 ppm DON with 50 mice per sex per dose level and fed for 2 years as a carcinogenicity study. DON was acquired from extraction and purification from field inoculated corn and from hydrolysis of 3-ADON

produced in liquid fungal culture. Average body weight of both sexes was reduced by 5 and 10 ppm dietary DON but only the males had reduced daily feed consumption at 5 and 10 ppm ($p < 0.001$) compared to controls. Of organ weight parameters, only the males exhibited significant reductions: liver at 5 ppm ($p < 0.05$); liver, spleen, and testes at 10 ppm ($p < 0.05$). Male mice also exhibited significant alterations of blood parameters: elevated RBC count, Hb, Hct, reticulocytes, and monocytes ($n = 7$, $p < 0.05$) at 1 ppm; reduced platelets and increased reticulocytes ($n = 9$, $p < 0.05$) at 5 ppm; increased eosinophils at 10 ppm ($n = 10$, $p < 0.05$). The females did not have any alterations of blood parameters nor did either sex have any alteration of bone marrow differentials. Females fed 10 ppm had significant increase in serum IgA ($n = 10$, $p < 0.05$) but no immunologic alterations were observed in males. Male mice exhibited significant negative correlations of several neoplastic lesions and DON dose but after Bonferroni adjustment for multiple comparisons the only significant effect was reduced incidence of hyperplastic nodules with increasing dose ($n = 48 - 50$, $p < 0.05$). Controlling for confounding factors was very strict in this study and the results reveal adequate samples to validate data. The authors report that although DON did not appear to have any inherent carcinogenic potential the reduction of neoplastic lesions was related to reduced body weight (20%). Little biological relevance was given to alterations in blood parameters and organ weights other than confirmed male sensitivity.

Dogs and cats may be exposed to DON or other mycotoxins as commercial diets have large proportions of corn or other cereal grains. The dietary toxicity was investigated utilizing a naturally contaminated wheat (37.2 mg DON/kg) mixed into nutritionally complete diets with 0, 1, 2, 4, 6, 8, or 10 mg/kg DON for dogs and cats (Hughes et al., 1999). Diets were extruded following DON incorporation and DON analysis following extrusion confirmed stability of toxin during processing. Doses of 8 ppm caused vomiting in 4/7 dogs exposed and 10 ppm caused vomiting in 9/13 dogs. Feed intake was reduced by 6 ppm or greater and several animals were removed from the study due to anorexia. Cats seemed to be slightly more tolerant as no vomiting was observed for doses less than 8 ppm although the 10 ppm pens had feed intake $46.7 \pm 10.1\%$ of the control group. The authors concluded that dogs may be as sensitive as pigs while cats may be more tolerant which may be due to differences in eating patterns, dogs being rapid consumers and cats being nibblers. It was also

suggested that rapid absorption and metabolism occurred following DON ingestion by dogs because emesis occurred quickly following meals and recovery was also rapid. Few animals were used in this study and the experimental unit was pens with variability of diet acceptance observed within pens. No other reports of toxicity in these species have been published but metabolic studies would define suitability of these species for other toxicity testing such as a non-rodent species for immunotoxicity testing.

There are no direct toxicity studies of the effect of DON on humans obviously but an epidemiological study was reported by Bhat et al. (1989). An incident in the Kashmir Valley of India occurred in the summer of 1987 in which several thousand people were affected by gastrointestinal disorder, initially not causing alarm but peaking interest when people of various socioeconomic statuses became affected indicating etiology other than sanitation related. House to house surveys were conducted in villages as well as surveys of consecutively clustered houses in randomly selected municipal wards. Of 150 families surveyed, 39 reported at least one member of the household affected by distinct gastrointestinal disease that previous summer. All 97 people affected reported feelings of fullness and mild to moderate abdominal pain 15 minutes to one hour after consuming their breakfast or evening snack that consisted of locally produced or homemade wheat bread. Other symptoms reported included throat irritation (63%), diarrhea (39%), vomiting (7%), blood in the stools (5%), and facial rash (2%). Increased incidence of upper respiratory tract infections was reported in children who had consumed the wheat bread for more than a week. Interviews also revealed lack of improvement with medical treatment and illnesses subsided when consumption of the wheat bread stopped. The investigation revealed that the spring of 1987 weather had been unseasonably rainy and most wheat marketed was mold-damaged with resulting flours of poor quality. Samples of flours and wheat in the local markets at the time of the investigation were collected, analyzed by TLC and found to contain DON (0.346 – 8.38 ppm, 11/17 positive), NIV (0.03 – 0.1 ppm, 2/19 positive), T-2 toxin (0.55 – 4 ppm, 4/19 positive), 3-ADON (0.6 – 2.4 ppm, 4/19 positive) but were negative for aflatoxins and ergot alkaloids. To evaluate the toxicity of the wheat, contaminated samples were extracted and incorporated into bread and fed to weanling canine pups at 200 µg/kg. All seven dogs fed the bread vomited within 3 hours whereas none of the four control pups showed any toxicity

signs. The authors estimated that mild toxicity was caused by the consumption of 30 to 150 g bread containing 0.34 mg/kg DON. While this was the only report of a dedicated investigation into an apparent DON related human toxicity event, the samples of wheat were collected at least two months after the outbreak, more contaminated product was most likely consumed. The estimated minimum toxic dose seemed low and the reasoning behind the calculation is not clearly stated. Interestingly, symptoms reported are consistent with animal toxicity studies and an increase in childhood upper respiratory infections after a week of consumption, although historically reported, many indicate immunosuppression doses of DON contributing to clinical illness.

DON was found in the urine of men affected by chronic idiopathic spastic paraparesis (CHISPA) with and without concurrent infection with human T-cell lymphotropic virus type-1 (HTLV-1) which occurs in hot humid climates (Leon-S et al., 1996). The finding was accidental in the initial patient but was further investigated in 12 patients with CHISPA + (positive for HTLV-1) and 3 patients CHISPA -. Urine (25 ml) was collected from these patients as well as from 4 apparently healthy and two patients with other neurologic disorders as controls. HPLC with fluorescence detection was used to analyze the urine for aflatoxins (B1, B2, G1, and G2), ochratoxin A, NIV, DON, and fumonisin B1. All the control samples were negative for all mycotoxins but DON was detected in 13 patients (0.3 to 12.4 μg/ 100 ml urine), 8 patients positive for fumonisin B1 (9.35 to 222.6 μg/ 100 ml), and 7 patients positive for NIV (0.22 to 1.3 μg/ 100 ml). Although no food samples were collected nor any statement about food consumption history, the authors made a comparison to an area of Japan that was endemic for CHISPA+ and consistently detected contamination of local foods with DON and NIV. Since metabolism of DON in humans was likely to be similar to other monogastric animals (pigs and dogs), the people who had mycotoxins in their urine would have consumed the contaminated foods within hours of urine collection and although it seems interesting that the control subjects did not have any detectable mycotoxins in their urine, it could have been by chance. Also the CHISPA subjects had been affected for at least 6.3 years so even though the finding is interesting, cause and effect can not be speculated.

Differences in species sensitivity to *in vivo* DON exposure are evident from numerous experimental trials. Swine appear to be the most sensitive with acute toxicity (emesis)

occurring at 0.1 mg/kg bw by i.p. injection or gavage (Forsyth et al., 1977). Reduced weight gain and feed intake occurs in pigs fed 4 ppm DON but not all altered physiologic parameters can be explained by energy restriction (Rotter et al., 1995). Dogs seem to be similarly sensitive with vomiting occurring at 6 ppm dietary DON or higher (Hughes et al., 1999) although cats tolerate a slightly higher dose (8 - 10 ppm), eating behaviors may explain this. Mice appear to be more sensitive than rats with a particular sensitivity of males (Iverson et al., 1995). Acute toxicity in rats may involve reactive oxygen species as toxic signs and alterations of free radical scavengers were prevented by supplementation with Se and Vitamins C and E (Rizzo et al., 1994). Mice have shown reduction of feed intake at doses of 2.5 ppm dietary DON (Robbana-Barnat et al., 1987) which may be related to the mechanism of reduced gastric emptying induced by doses as low as 50 μg/kg bw (Fioramonti et al., 1993). This mechanism may also be functioning in humans as the most common complaint was feeling full following a suspected DON toxicity investigation (Bhat et al., 1989). Anecdotal evidence for immunotoxicity in humans was reported in the former study with increased incidence of upper respiratory tract infections in sub-acutely exposed children and the finding of three mycotoxins in the urine of men with CHISPA with or without concurrent HTLV-1 infections (Leon-S et al., 1996). *In vivo* data indicated sensitivity to DON toxicity was dependent on species, strain, sex, route of administration and length of exposure. Studies should be interpreted with these variables in mind.

In vitro Toxicity other than Immunological

Evaluation of toxicity of DON exposure to primary cells or cell lines, outside of immune related cells has been limited although have helped to characterize mechanisms of *in vivo* toxicity. Studies reviewed here discuss effects on hepatocytes and colonic cell lines. Bradlaw et al. (1985) investigated the effect of purified DON on unscheduled DNA synthesis (UDS) as a measure of genotoxicity in primary rat hepatocytes. Primary hepatocyte cultures were prepared from adult male Sprague-Dawley rats by collogenase perfusion of fresh livers and the viability of acquired hepatocytes was assessed by Trypan Blue exclusion (82 – 86%). Cells were cultured in Williams Medium E with 10 μCi of [Me-^{3}H]thymidine per ml media and DON 0.1 to 1000 μg/ml media, 2-acetylaminofluorene (2-AAF) 22.3 and 223 ng/ml media as positive control, or DMSO vehicle controls for 19 hours. UDS was measured by

counting the number of cells in replicative DNA synthesis per 1000 cells with replication assignment based on greater than 100 grains, incorporation of [Me-^{3}H]thymidine detected by autoradiographic development, per nucleus. Background counts were consistent with media controls and cells were deemed metabolically active due to significantly increased UDS in 2-AAF exposed cells ($p < 0.01$), cellular activation required for chemically induced DNA damage. DON did not cause any alteration in UDS at 13 doses in range given above. Three replicates of each dose were tested and adequate controls were included to validate the data. No statement of prior diet, acclimation period, or number of rats used (pooled?) was stated.

Additional genotoxicity studies were conducted with primary rat hepatocyte exposure to DON and the Ames test with *Salmonella typhimurium* strains TA98 and TA100 with and without S9, hepatic microsomal preparation for test chemical activation (Knasmüller et al., 1997). Hepatocytes, isolated from female Fischer 344 rats which had been on lab chow and acclimated for at least 2 weeks, were obtained by collagenase perfusion and incubated in minimal essential media with the toxins added to the media dissolved in DMSO for 3 hours, 3 plates per concentration. Media was exchanged for fresh and cells were stimulated with epidermal growth factor (40 ng/ml) for 48 hours, cells fixed and stained to determine the mitotic index and micronuclei per 1000 cells. Chromosomal aberrations were also determined from one of the replicate plates. The Ames test was conducted as plate incorporation assays mixing 0.1 ml stationary phase bacteria cells, 0.1 ml of various concentration test compounds, ± 0.5 ml S9 (Aroclor 1254 induced rat) in 2.0 ml top agar on selective media plates. After 48 hours incubation colonies were counted, increased colonies compared to media controls indicates mutation induction by the test substance. DON (0.7 – 500 µg/plate, 3:1 dilutions, 7 levels), FB1 and moniliformin (MON), at any concentration tested, did not induce mutation in the Ames test. DON caused reduced mitotic index ($p < 0.05$) at 10 µg/ml and cytotoxicity at 1000 µg/ml in expose hepatocyte cultures. Hepatocyte micronuclei were more frequently observed only at 10 ng DON/ml media ($p < 0.05$), although also elevated at 1 and 100 ng/ml, and increased chromosomal aberrations were observed in these cells at 1.0 ($p < 0.05$) and 10.0 µg ($p < 0.005$) DON/ ml media. Generally the other two mycotoxins, same dose range as DON, caused a similar pattern of reduced mitotic index at higher doses, increased frequency of micronuclei at lower doses and

chromosomal aberrations at higher doses. FB1 and MON are not related to DON and exhibit difference mechanisms of action although FB1 has been found in DON contaminated corn samples. DON was not genotoxic in the Ames test, but evidence for DNA damage was observed in rat hepatocytes, which has not been observed *in vivo*. The authors suggested a possible connection with free radical production and protective effect of antioxidants reported by Rizzo et al. (1994) although no evidence of carcinogenesis occurred after two years of dietary exposure to mice (Iverson et al., 1995). Adequate controls and appropriate statistical analysis was included in this study. Due to the lack of evidence for a complementary *in vivo* toxic effect of DON exposure, further research to characterize this effect has not been conducted and does not seem warranted.

Dietary DON exposure has caused gross and histopathologic changes in gastric epithelium. *In vitro* effects of DON exposure to human colonic adenocarcinoma cell lines (Caco-2 and T84) was assessed by measurements to evaluate cell growth (scanning electron microscopy), differentiation (brush border membrane associated hydrolases alkaline phosphatase and sucrase-isomaltase), and tight junction integrity (lucifer yellow dye exclusion and transepithelial electrical measurements, TEER) (Kasuga et al., 1998). The cells were exposed to DON in the media (0, 50, 100 or 200 ng/ml) upon seeding and at the same concentration with subsequent media addition. Cellular confluence of Caco-2 cells was reached after 12 days of incubation at any DON concentration but widening of cellular junctions and elongation of microvilli was observed at 100 ng/ml and became more pronounced at 200 ng/ml although most microvilli were absent or shortened. The loss of tight junction integrity was quantified by increased lucifer yellow dye permeability ($p < 0.05$) across the cell lawn at 100 ng/ml for Caco-2 cells and at 200 ng/ml for T84 cells. As an indicator of increased ion permeability across the cell monolayer, TEER was significantly decreased ($p < 0.05$) at 50, 100 and 200 ng/ml for Caco-2 and at 100 and 200 ng/ml for T84 cells. Differentiation of Caco-2 cells was also affected as alkaline phosphatase activity was reduced ($p < 0.05$) by 100 and 200 ng DON/ ml media from the 6^{th} to the 15^{th} day of culture but sucrase-isomaltase activity was decreased ($p < 0.01$) by 50 and 100 ng DON/ ml at 10, 15 and 20 days of culture. The authors chose DON doses below preliminarily determined acute cytotoxicity dose so that cells would reach confluence. The authors suggested that the results

of this study may provide evidence for lesions observed following *in vivo* DON exposure since enterocyte differentiation was required as a barrier to infectious agents. Enterocytes have high turnover thus DON effects on proliferation and differentiation may be important. It would also be important to investigate the effects of DON exposure in a mature monolayer and measure the same parameters.

Two types of cells which may be exposed to high DON levels upon ingestion were investigated for dose-responses, primary rat hepatocytes and immortalized colonic cell lines. DON did not cause increased unscheduled DNA synthesis (Bradlaw et al., 1985) but was determined to cause chromosomal aberrations at higher doses (Knasmüller et al., 1997) in rat hepatocytes. Proliferation and differentiation of Caco-2 and T84 cell lines was altered resulting in loss of tight junctions, 50 – 200 ng DON/ml media, and monolayer integrity (Kasuga et al., 1998). As liver toxicity has not been observed *in vivo*, results with primary hepatocytes confirmed the lack of DON toxicity in this organ. Gastrointestinal epithelium toxicity has been observed *in vivo* in animals and anecdotally in humans (Bhat et al., 1989) therefore it was important to investigate the impact of DON on these cell types. These cells were sensitive to doses that could be achieved with oral ingestion of DON contaminated foods. Loss of tight junctions and poor brush border development could contribute to diarrhea, necrosis of gastric epithelium either due to barrier loss and acidic gastric content exposure to deeper tissues, increased absorption of toxic substances from the lumen, lack of absorption of nutrients, or opportunity for microbial invasion.

Immunotoxicity

Xenobiotic effects on the immune system have been recognized for a long time. Importance as a distinct target organ with specific protocols for testing a chemical for immunotoxicity has only occurred within the last twenty years. Standardization of the descriptive toxicological studies to evaluate effect on the immune system was attempted through a committee effort for the National Toxicology Program (Luster et al., 1988). The goal was to develop and validate a flexible yet sensitive and specific protocol for identification of immunotoxic compounds. The committee proposed a tiered approach based on a series of screening assays following appropriate exposure routes and times in rodents and the option of further immunologic evaluation if warranted by results of the first round of

assays. Tier I evaluates both innate, natural killer cell cytotoxicity and lymphocyte proliferation, and acquired immunity, IgM antibody plaque-forming cells to sheep red blood cells, as well as general body conditions and immunohistopathology. Tier II assays would be dictated by the type of immune alteration expressed in the first tier to further characterize the mechanism of compound immunomodulation. Tier II assays could include spleen cell phenotyping and enumeration, IgG antibody response to SRBC, delayed hypersensitivity response, function of peritoneal macrophages, and two or three host resistance challenge models. The authors indicated that of the compounds tested under this scheme, none had shown alterations in Tier II assays without some alteration of Tier I. The protocol design for Tier I assays were intended to be incorporated into a standard carcinogenesis protocol with dosing based on intended or suspected human exposures. Mice were chosen as the species of choice due to more historical control data, availability of immunological data, large background of genetic data, and validated models of susceptibility for host resistance evaluation. Since the goal of this committee was to develop standardized guidelines, the immune assays were evaluated by several factors prior to inclusion: intra- and interlaboratory reproducibility, accuracy based on optimal historical data, assay sensitivity determined by dose-response curves, and predictability based on correlations with other immune function tests. These criteria allowed a grading system for comparing immunotoxicants as well as data validity for human risk assessment. Initial validation studies were conducted for five compounds in three labs and it was observed that reproducibility and accuracy improved as the laboratories developed expertise and that variability decreased since a standardized protocol was followed. Considering the reserve capacity of the immune system and the pleotrophy of resistance to invading challenges, the validation studies were able to determine that some Tier I assays were better able to predict and correlate with appropriate host challenge studies. For example, an impaired induction of plaque-forming cells to sRBC and suppressed lymphocyte proliferation correlated well with impaired increased mortality in *Plasmodium* or influenza challenges. The validation studies were further able to establish control values for normal animals that will add to the historical database. As the initial guidelines for a standardized protocol, all of the measures were incorporated for validation.

Positive controls were included and variability observed improved with repetition. The tiered approach saves resources by being comprehensive initially and more focused when required.

NTP guidelines were followed for several years to develop a database of 51 chemicals. This database was used to evaluate the sensitivity and predictability of the assays (Luster et al., 1992) for immunotoxicity. To define a chemical as immunotoxic a liberal definition of immunotoxicity was derived. The chemical either produced a significant ($p < 0.05$) dose-response effect, excluding body weight alteration, in one or more immune function assays or significantly altered two or more tests at the highest dose tested. The authors recognized bias in the analysis produced by attempting to determine immunotoxicity from conducting immune function assays but were not able to correct for this. Further validity to the analysis was achieved by independent review by five outside experts. More conservative statistical approach was used to analyze for correlation between altered immune function tests and non-immune endpoints such as carcinogenesis. Sensitivity, specificity, and concordance (correctly predicting immunotoxicity from immune function tests) were determined from two-by-two contingency tables using results of the immune function assay on one axis and the chemical's immunotoxicity classification on the other axis. Thus the authors were able to statistically define the relationship of one immune function assay to immunotoxicity of the chemical in question. It was determined from this analysis that the T-cell dependent sRBC PFC assay (78%) or the splenic lymphocyte population phenotyping (83%) had the highest concordance to predict an immunotoxic outcome. None of the other individual assays alone were good predictors of immunotoxicity and generally the decrease in concordance was due to lack of sensitivity. Concordance could be improved (> 90%) if two assays were conducted, particularly if one of them was the PFC or lymphocyte phenotyping and a few combinations of three assays could achieve 100%. Concordance was also evaluated between immunotoxicity and carcinogenicity. The database of chemicals was reduced due to lack of data for some compounds but it was found that of the immunotoxic compounds 100% were carcinogenic (19/19) and of those in the database not immunotoxic only 63% were carcinogenic (5/8). The authors recognized the limited number of compounds included in this analysis, due to data completeness, and recommended a larger database to make further conclusions. Conversely genotoxicity and immunotoxicity had poor

concordance (48 to 69%) and no relationship could be defined. An explanation for the power of the PFC assay and lymphocyte phenotyping was minimization of reversibility due to proximity of assay to the *in vivo* function. In other words, the cells have had less influence from media and time for the effects of the test chemical to wear off. Additionally, multiple processes are involved in the functional aspects of these cells (antigen processing and presentation, synthesis and release of cytokines, cell proliferation, differentiation and secretion) so alterations in function are more closely related to *in vivo* effects. One problem with lymphocyte phenotypic enumeration discussed by the authors was interindividual variability in the human population that does not exist to the same extent in inbred mice. "Normal" human databases will most likely need to be developed as sources for data comparison. This comprehensive study provided substantial validity to an immune function testing protocol. They had a database of 51 different chemicals to compare in the specificity, sensitivity, and concordance of the assay results and immunotoxicity of the compound. Even though the authors admit that a liberal statistical definition of immunotoxicity was applied, a few assays reliably predict immunotoxicity. This does not mean that fewer assays should be conducted in the first tier of a chemical's immunotoxicity evaluation but that if positive results are found for PFCs or alterations of lymphocyte phenotypes, the chemical is more likely to be immunotoxic.

Predicting chemical immunotoxicity through immune assays only tells part of the story. Resulting alteration in susceptibility to tumors or infectious agents in the face of the complexity of the immune system is much more difficult to predict. The database developed through the NTP guideline development and validation was used to try to define the relationship between immune function assays and host resistance assays (Luster et al., 1993). Using the established database and two additional dose-response trials with the prototypic immunotoxicant cyclophosphamide, statistical analysis was conducted to determine the specificity, sensitivity and concordance between immune tests and host resistance assays in mice. They found overall 100% specificity but only 50% sensitivity resulting in low concordance. Positive immune assays would only accurately predict reduced host immunity 50% of the time notably due to the liberal definition of immunotoxicity. When correlations between immune function tests and host resistance assays did occur, it was due to related

immune mechanisms. For example, suppressed PFCs were correlated with increased susceptibility to *Listeria*, *Staphylococci*, or *Plasmodium* because specific antibody production is needed for resistance to these organisms. NK cytotoxicity was not as reliable, being correlated to altered resistance to *Listeria* and PYB6 tumor formation but not B16F10 melanomas. An important finding in this study was that host resistance suppression only occurred if there had also been immune function test alterations. The host resistance assays need to be selected with some prior knowledge of mechanism of immune function alteration to get the best information for characterization of the effects of the chemical being tested. Further, the results of the host resistance assay would depend on the virulence and dose of the challenge tumor or organism. The challenge may be able to overwhelm any individual in the population even with apparently adequate immunity or conversely a small change in immune function may have a large impact on host resistance depending on the challenge. Using additional cyclophosphamide exposure assays with a large range of doses (25 to 200 mg/kg bw in 25 mg increments), the authors were able to develop models of the relationships between the immune test and host resistance assays. In most cases the relationships fit linear or linear-quadratic regression models. Thus a certain percent change from control in an immune assay would result in a similar percent change in host resistance although variability was reflected in wide confidence limits for some assays. For example a 10% decrease in resistance to *S. pneumoniae* would be reflected as 11.6% (4.8 – 40.7%, 95% confidence interval) suppression of PFCs. Additionally, the models developed for cyclophosphamide generally did not fit any of the other 18 compounds with enough data for comparison. It could be concluded that each compound affects the immune system differently with a different relationship between the effects on a measured immune test and the *in vivo* host resistance. This study brought together a substantial amount of data on a wide variety of compounds and provided validity for the guidelines proposed for immunotoxicity testing of compounds. Even though a liberal definition of 'immunotoxic' was applied to chemicals resulting in altered immune function tests, linear relationships occurred between immune function tests and host resistance assays. In a large population where there would be variability in immunotoxicant exposure and infectious agent exposure, the importance of dose-response relationships becomes vital to understanding the risk of a population.

Most investigations of immunotoxicity currently include cytokine assessments. Cytokines are glycoproteins produced by cells in the immune system upon activation that provide signals to other cells. There is some cell specificity of cytokine production but activity tends to be redundant and pleiomorphic. For example, interleukin-4 (IL-4) is produced by activated T-helper cells and acts to inhibit cellular immunity and promote antibody production by B-cells but IL-10 also has a similar function. Due to the complexity of the cytokine system, measuring an alteration in only one or two of these proteins may provide evidence for a mechanism of immunotoxicity but does not define a chemical as immunotoxic. House (1999) reviewed the significance and complexities of cytokine assessment. Cytokines are measured by ELISA or specific cytokine-sensitive cell lines. Generally cytokines are not released without cell activation and most assays use mitogen stimulated immune cells as the cytokine source although some can be measured directly in serum. Serum levels may not be accurate because the half-lives of some cytokines are very short (minutes), sensitivity of the assays requires superphysiological concentrations, and 'normal' levels have not been established. Immune cell activation to measure cytokines following *in vivo* chemical exposure has the complication of potential reversal of toxic mechanism due to length of incubation. *In vitro* exposure to the test chemical may provide a more controlled environment for alteration in cytokine production but without the interaction of the whole immune system and whole body effects, relevance of changes in cytokine production may be more difficult to interpret. The mouse cytokine system was the most understood and has the largest number of assays available but there are species specific cytokine functions that need to be considered if extrapolating cytokine data to another species. For example, IL-5 has different functions in the rodent and humans. An additional approach to assessing cytokines is molecular biology techniques that measure specific mRNA which was being reported more frequently in the literature. These techniques may be very sensitive and specific for increased expression of mRNA for a cytokine but functional molecules are not measured thus the biological significance may not be correlated to the measured alteration.

While the preceding series of reports outline a valid, comprehensive approach to immunotoxicity testing in experimental animals, the ultimate goal is to accurately extrapolate

this information to risk assessment of human exposures to immunotoxic compounds. The ideal situation would be to conduct these studies in exposed humans but the drawbacks of this are obvious. Tryphonas (2001) outlined the practicalities of performing immunotoxicity evaluations in humans. This review related that even though the experimental animal database is extensive for immunotoxicity of chemicals, human data is limited and incomplete. Of data that has been obtained in human studies, it was usually following occupational or accidental exposures that were difficult to characterize and often appropriate unexposed controls were not obtained. The author lists several published screening protocols for assessing immunotoxicity in humans but related that no epidemiological study has used any of these in its entirety. Immune system assays described by the author that can be conducted in humans were similar to ones recommended by Luster et al. (1988) with minor modifications. WBC in humans has more value than in animals since it exhibits little individual variability over time although can be significantly influenced by factors such as age, sex, race, stress, pregnancy, nutritional status, smoking, or coexistent infections. 'Normal' values used for comparison should be age and sex matched minimally. Similarly for peripheral blood cell phenotyping, age was a particularly important control factor since total lymphocyte counts change in age groups, especially as lymphocyte populations are maturing in childhood. Quantification of basal serum immunoglobulin classes had limited value as predictor of susceptibility of infection but antibody production to specific antibody had predictive value similar to rodent studies. Tryphonas (2001) recommended two antigens that could be safely administered to people, bacteriophage phiX174 and keyhole limpet hemocyanin (KLH), since they have not been observed to cause any adverse effects with repeated injections and were not likely to have been encounter by the immune system previously. Assays to assess cellular immunity include mitogenic response of peripheral blood lymphocytes and measurement of inherent and stimulated cytokine release from these cells, delayed hypersensitivity responses to a battery of skin injected test antigens, and cytotoxicity of NK cells from peripheral blood. All of these assays have large databases of rodent data and have been validated repeatedly but interpretation of results would be complicated by large interindividual variability. The author cautioned that any changes observed in immune function must be correlated with blood/adipose tissue levels of the

toxicant in question. This review provided information on clinically relevant immune assays that can be conducted in humans when investigating an immunotoxicant and some insight into the considerations important to study design. The most restrictive aspect of conducting a study would be the number of subjects required to achieve adequate power for valid interpretation of acquired data and appropriate controls in order to detect effects of exposure.

Immunotoxicity evaluation of any compound should involve a battery of assays assessing the major functions of the immune system as well as cells available to mediate these functions. Luster et al. (1988) found strong concordance between alteration in production of PFC in response to sRBC and spleen cell phenotyping and accurate prediction of chemical specific immunotoxicity. Inhibited Tier I functions warrant further investigation by Tier II assessments which include host resistance assays. Alterations in host resistance assays were not found without immune function alteration (Luster et al., 1993) and tend to have a linear regression relationship. Cytokine measurements from serum or *ex vivo* can suggest mechanisms of immunotoxicity but House (1999) cautioned that many cytokines are labile and functionally overlap. Work involving cytokine mRNA expression has limited utility if xenobiotic toxicity occurs at transcription or post-transcriptional. Studies to investigate immunotoxicity in humans was described as more complicated (Tryphonas, 2001) due to confounding factors, limited exposure regimes available, careful selection of appropriate 'normal' controls, and interindividual variability. Study of xenobiotic effects on the immune system was a relatively new specialized avenue of investigation but has become more prevalent as the incidence of childhood asthmas and concerns for hypersensitivity reactions to new protein antigens introduced by genetically engineered foods. Difficulties arise from investigation of a xenobiotic's potential for immunotoxic effects in the controlled environment of the laboratory and extrapolation of that data to human risk assessment. Not only is the chemical of interest not the only chemical to which people are exposed but humans expose themselves to so many other immunomodulating factors (smoking, nutrition, exercise, stress) that finding anecdotal evidence for immunotoxicity in a human population warrants further investigation. Interactive factors that may expose a high-risk population, particularly if control measures can be instituted, should be determined by a comprehensive systemic approach as outlined by the previous reports.

Immunomodulation by DON in vivo

In vivo immunotoxicity studies have been conducted almost exclusively in mice with complementary studies in numerous cell types *in vitro* to further characterize effects of DON. An early study evaluated the humoral dose-response of weanling male Swiss-Webster mice to once a day gavage with DON for 5 weeks at doses of 0.75, 2.5 and 7.5 mg/ kg bw with the inclusion of solvent and untreated controls (12 per group at initiation of study) (Tryphonas et al., 1984). All of the animals died that were given the highest dose and 4 animals died in the 2.5 mg/kg group, all due to the treatment. Antibody titers to sRBCs (given i.p. 5 days prior to termination) were significantly reduced in both remaining dose groups compared to vehicle controls ($p < 0.004$), PFCs were reduced but statistical significance was not met. Thymic weight reduction was dose-responsive ($p < 0.001$) but spleen weight was only reduced by 0.75 mg/kg bw ($p < 0.02$). Serum protein profiles by electrophoresis was altered significantly by the lower dose with an increase in albumin ($p < 0.01$) and reduction in α-2 globulin ($p < 0.001$) resulting in a significant elevation of albumin/globulin ratio ($p < 0.01$) compared to the vehicle controls. This study was reported to be preliminary data but there was apparent vehicle effects, most likely the result of handling stress to gavage, that reduced (not statistically significant) spleen and thymus weights as well as PFC and antibody titers. Although DON treated groups were compared to the vehicle control animals, this chronic stress effect may have influenced the true immunotoxic effect of DON.

In follow-up experiments (Tryphonas et al., 1986), weanling male Swiss-Webster mice were fed 0, 0.25, 0.5, or 1.0 mg DON/kg bw which was equivalent to 0, 1, 2, or 4 ppm DON in the diet. After 5 weeks exposure (starting at 3 weeks of age) the only significant effects were reduced serum α-2 globulin in all DON fed groups ($p < 0.035$, $n = 10$) and increased albumin in the 1.0 mg/kg group ($p < 0.048$). To investigate the reversibility of dietary DON effects, the protocol from the first experiment was repeated ($n = 6$) but at the end of the 5 week feeding, all groups were fed the control diet for 40 days before termination. Serum protein effects were not reversible with elevated albumin in all DON dose groups ($p < 0.041$) and reduced α-1 globulin in the 1.0 mg/kg bw group. Serum antibody titers, PFCs, spleen or thymus weights were not significantly different. A *Listeria* challenge study was conducted on a third set of mice fed the same DON diet above ($n = 10$)

injecting i.v. 8.4 X 10^4 colony forming bacteria (LD_{20-30}) diluted in saline and mortality was recorded over 18 days. It was not stated if the experimental diets were continued. Even though a similar number of animals died per group (3 from each control, 0.25, and 0.5 mg/kg bw groups; 2 from 1.0 mg/kg bw group) DON caused a dose-response inverse relationship to time of death (7.3, 6.3, 5.5, 5.3 days for control, 0.25, 0.5, and 1.0 mg/kg respectively). One final study was conducted using the same protocol but after 5 weeks feeding the test diets (n = 6), splenocyte proliferation assays were conducted using the mitogens Con-A, PHA-P, and LPS. Cells were incubated with and without mitogen for 48 hours prior to addition of tritiated thymidine and incubation for an additional 16 hours. Although no significant differences were observed for any media controls or mitogen stimulated splenocyte cultures, the data suggested a stimulatory effect of DON with or without Con-A and PHA-P. The authors also reported significantly reduced feed consumption for mice fed 1.0 mg/kg bw compared to controls ($p < 0.001$) although body weight gains fluctuated for all groups since all four experiments were pooled for this comparison. The authors estimated the no-effect level of DON exposure resulting in immunomodulation to be between 0.25 and 0.5 mg/kg bw per day. This seems reasonable as sub-chronic dietary studies observed reduced weight gain and feed intake at 2 ppm dietary DON (Forsell et al., 1986).

As previously indicated, multiple mycotoxins may occur in one food source, particularly DON and ZEA. Pestka et al. (1987) investigated the immunological effects of dietary DON and ZEA separately and in combination in B6C3F1 weanling female mice. Purified DON and ZEA from culture were incorporated into AIN-76A semi-purified diet resulting in diets containing: 0.5 ppm DON, 5 ppm DON, 25 ppm DON, 10 ppm ZEA, or 5 ppm DON + 10 ppm ZEA. Additional pair fed control groups were included that were given the same amount of feed consumed by the 5 ppm and 25 ppm DON groups. Animals were fed for either 2 weeks or 8 weeks then immune function assays were conducted: splenic clearance of *Listeria monocytogenes* injected i.v. (diet treatments 0.5 DON, 5.0 DON, 10.0 ZEA, 0.5 DON + 10.0 ZEA and 5.0 DON + 10.0 ZEA), PFC response to sRBC priming (diet treatments 5.0 DON, 25.0 DON, 10.0 ZEA, 5.0 DON + 10.0 ZEA), and delayed hypersensitivity to KLH injected in footpad, same diet treatments as PFC. Dietary exposure to mycotoxins for two weeks resulted in significantly delayed splenic clearance of *Listeria* (1

X 10^4 CFU) in the DON (5.0) + ZEA (10.0) group 1 day after injection ($p < 0.05$, $n = 5$) compared to *ad libitum* controls which continued 4 days post challenge, and DON (0.5) + ZEA (10) group had significantly delayed clearance compared to DON (0.5) ($p < 0.05$, $n = 5$) at 4 days post challenge. The groups fed DON (25 ppm), DON (25 ppm) + ZEA (10 ppm), and ZEA (10 ppm) had significantly delayed clearance (5 X 10^4 CFU) compared to *ad libitum* controls ($p < 0.01$, $n = 9$) but not restricted controls 4 days post challenge. There were no differences detected after 8 week dietary treatment for either challenge dose or time post challenge. Humoral immunity was also effected by dietary treatments resulting in significantly reduced sRBC PFC/ spleen in DON (25 ppm) ($n = 10$) compared to *ad libitum* controls ($n = 24$) ($p < 0.01$) while the pair-fed controls consuming equal diet to the DON (25 ppm) group were the only group having reduced total spleen cell counts compared to *ad libitum* controls ($p < 0.01$) after 2 weeks feeding. There appeared to be a mean increase in PFC/ per 10^6 spleen cells in the DON (5.0 ppm) group but the SEM was 50% of the mean. After 8 weeks exposure reduced total spleen cells occurred in pair fed controls (25 ppm) and DON (25 ppm) groups ($p < 0.01$) but DON (5.0) + ZEA (10.0) had significantly higher cell numbers ($p < 0.05$) compared to *ad libitum* controls. Sheep RBC PFC per 10^6 spleen cells was increased by restricted feeding ($p < 0.01$) compared to *ad libitum* controls whereas PFC formation after DON exposure (25 ppm) was significantly reduced when compared to pair fed controls ($p < 0.01$). Delayed hypersensitivity to KLH was reduced by 3 week feeding of DON (25 ppm) compared to *ad libitum* or pair fed controls ($p < 0.01$) and DON (25 ppm) + ZEA (10 ppm) ($p < 0.05$). No treatment effects were observed after 8 weeks feeding. The authors summarized the effect of DON and ZEA on splenic *Listeria* clearance based on the two experiments but one important factor that was omitted in the discussion was the difference in challenge dose. Delayed clearance observed when mice were challenged with 1 X 10^4 CFU was not observed at 5 times the dose with a larger sample size (5 vs. 8 animals) and 25 ppm DON fed mice were not challenged with the lower bacterial dose. Although all three immune function assays conducted were affected by dietary mycotoxin exposure, dose of agent challenge and length of mycotoxin exposure were important parameters elucidated by this study. Feed refusal as a consequence of mycotoxin exposure may contribute to suppressed immune function but since these authors did not report the actual feed intake, it

was difficult to assess the extent of feed intake reduction. Mechanism of adaptation to chronic mycotoxin exposure has not been investigated and could prove to be one explanation for lack of toxicological incidence in populations where mycotoxins are found in food supplies chronically.

General immunosuppressive effect of DON in weanling male BALB/c mice exposed to daily i.p. injections of 5 mg DON/kg bw in DMSO-PBS (1:9 vol/vol) or dietary DON (purified from culture) fed at 2.5 to 100 ppm was also determined (Robbana-Barnat et al., 1988). *Ad libitum* and pair fed controls, to what dose of DON was not indicated, were included. Injection of DON, although tolerated well, caused reduced thymus/ body weight ratio ($p < 0.01$, $n = 8$) compared to *ad libitum* controls but did not result in a significant reduction of anti-sRBC antibody titer. In the same experiment mice fed 50 ppm dietary DON (5 mg/kg bw equivalent) thymus to bw ratio was significantly reduced ($p < 0.001$) and antibody titers were reduced (11 ± 1.8 vs. 208 ± 48, $p < 0.001$) compared to *ad libitum* controls. Dietary restriction had no effect on these two parameters. When lower doses were fed, 10 ppm was the threshold of significant reduction of: anti-sRBC antibody titers at 1 ($n = 8$) and 2 weeks feeding ($n = 5$) ($p < 0.05$); thymus: bw ratio ($p < 0.01$) after 1 week; [^{3}H]thymidine incorporation in PHA stimulated spleen cells ($p < 0.001$), LPS stimulated spleen cells ($p < 0.001$), and PHA stimulated thymic cells ($p < 0.001$) after 1 week feeding compared to pair-fed controls. Histological examination of mice fed 10ppm DON revealed marked thymic atrophy to the extent of inability to distinguish between cortex and medulla. Although the aims of this study were the immunosuppressive responses of these animals, inclusion of data for dietary intake and immune responses of *ad libitum* controls would have been helpful to assess any influences of dietary restriction. Mice fed 1 ppm for 2 weeks were included in the sRBC data along with 2.5 and 5 ppm. These doses did cause some suppression of antibody titers and proliferative responses of immune cells. Although the authors mentioned differences in response recorded between the two routes of exposure initially tested, lower doses were not tested by i.p. injection and 5 mg/kg bw was half of the dose that killed all of the mice in their dietary study (100 ppm). These animals were also quite young (4-6 weeks old) and diet was not specified. This study omitted data that would have helped clarify the response observed but it did provide evidence of immunosuppression

in BALB/c male mice may occur at doses consistent with other mouse strains. Previously this investigator had found BALB/c male mice to be sensitive to dietary DON with feed intake reduction at 2.5 ppm (Robbana-Barnat et al., 1987). Investigation of other experimental parameters that can alter the immune response in these animals such as age (immaturity) or environmental stressors (singly vs. group housed) may provide additional insight into differences between studies with the same animal strain.

As discussed in the general introduction to immunotoxicity above, host resistance assays are more definitive assessment of the effect of immunomodulation *in vivo*. Two studies have investigated specific responses to different immunological insults in the face of DON exposure. Atroshi et al. (1994) tested the hypothesis that acute or sub-acute exposure to DON would influence the incidence and severity of mastitis in mice infected with *Staphylococcus hyicus* or *Mycobacterium avium* as a model for bovine mastitis. Lactating (first litter) NMRI mice were gavaged with 12.5 mg/kg bw once or 6.25 mg/kg bw once a day for 7 days, last treatment 6 hours prior to infecting two mammary glands (R4 and L4) with *S. hyicus* (2.5 X 10^8 CFU) or *M. avium* (3 X 10^6 CFU). Due to differing growth characteristics of these organisms, *S. hyicus* infected mice were examined for effects 48 hours after infection while 17 days was the period for *M. avium* infected animals. There were significantly decreased number of animals that appeared ill ($p < 0.01$) and severity of infection in the glands was reduced but inflammation was still evident when infected with *S. hyicus*. *M. avium* infected animals treated with a single dose of DON were not different from controls for signs of illness (80% ill) or severity of infection in glands. Animals that had been dosed with DON for a week before *S. hyicus* infection were not different from controls for illness but the severity of mammary gland infection was significantly reduced ($p < 0.05$) as well as number of CFU recovered from the infected glands. Serum IgA (ELISA analysis) was significantly elevated in both infection groups ($p < 0.05$) following a single oral dose of DON and after a week of treatment serum IgA, IgG, and IgM were all significantly elevated ($p < 0.05$) compared infected controls. Although the authors attempt to extrapolate this data to mastitis in cows, DON bioavailability differences between these two species were not considered and likely would invalidate mice as a model for cattle. IgA stimulation appeared to be independent of infecting organism but non-infected DON treated controls were not

included in the study to distinguish this issue. Although all classes of Ig were elevated by one week DON treatment, 48 hours would not be adequate time to influence a primary immune response to *S. hyicus* unless a secondary response was observed due to prior exposure to this organism or one with cross-reactivity. Again another control group would have provided clarity to the actual interaction between oral DON exposure and a mastitic organism.

Hara-Kudo et al. (1996) investigated the immune response of male BALB/c mice to 2 ppm DON (in water) two weeks prior to and concurrent with oral intubation of *Salmonella enteritidis* (3 X 10^6 CFU). Mice were euthanized 3, 5 and 7 (n = 4 mice per treatment per time period) days after *S. enteritidis* exposure with continuous DON exposure and bacteria counts were conducted in the mesenteric lymph nodes (LN), liver and spleen. DON treated, non-infected mice did not exhibit reduced weight gain, water intake, or alteration of liver or spleen weight. Bacterial counts were increased in mesenteric LN 3 days after infection in DON-treated mice compared to infected controls but were not different at 5 or 7 days after infection. Spleen bacterial counts were significantly higher at 7 days post-infection for the DON-treated mice ($p < 0.05$). Even though appropriate controls were included, it appeared that statistical analysis was conducted with each animal as an experimental unit while animals were housed 4 to a cage with a common water source. Details on diet composition and feed intake were also not reported. This report indicated an inhibitory effect of ingested low dose DON on clearance of a commonly encountered enteric bacteria but it would be useful to repeat this study with dietary exposure and measurement of immune function such as bacteria specific antibody responses and blood and spleen cellularity.

Investigations into the immunologic effects of DON have become more specific and mechanistic in the last decade. Two reports with relevance to our research will be reviewed here that measured *in vivo* cytokine release from orally DON exposed mice. Zhou et al. (1997) measured a battery of cytokine mRNA expressed in the spleen and Peyer's patches (PP) of male B6C3F1 mice following a single oral dose of 0, 0.1, 0.5, 1.0, 5.0, or 25.0 mg/kg bw (n = 3) DON dissolved in carbonate-bicarbonate buffer. Two hours after treatment tissue cytokine mRNA was extracted, measured by reverse transcriptase-polymerase chain reaction (RT-PCR) with Southern blot analysis. Serum cytokine measurements (ELISA) were made from mice exposed to a single oral dose of 25 mg/kg bw and bled 3 hours later. Elevations in

expressed mRNA due to 5 or 25 mg/kg bw DON dose was observed for TNF-α, IL-1β, IL-6, IFN-γ, IL-2, IL-12 p40, IL-4, IL-10 in the spleen and to a lesser extent in Peyer's patches. Lower doses did not significantly alter cytokine expression. TGF- β and IL-5 were constitutively expressed and were not affected by treatments, no other cytokines were measured. TNF-α, IL-6, IFN-γ were the only cytokines found in detectable levels in the serum of DON dosed mice while no detectable levels were found in vehicle control mice. A protocol detail of concern was the dissolution of DON in a carbonate-bicarbonate buffer (pH 9.6). DON is sensitive to alkalinity (Wolf and Bullerman, 1998; Abbas et al., 1988) and depending on the time in this solution, some compound degradation could have occurred lowering the effective dose. Although all animals were handled similarly, the stress of food and water removal 2 hours prior to gavage, with euthanasia 2 or 3 hours later (unspecified route) could have caused a stress hormone spike which may have altered cytokine mRNA expression. Inclusion of an untreated control group would have ruled out this potentially confounding factor. This study is reviewed here because it is the first report of dose-response cytokine effects although extrapolation of this data to our research interest is limited.

In a follow-up study, Zhou et al. (1998) investigated sub-chronic dietary DON exposure following a similar protocol for cytokine mRNA described above, although serum cytokines were not measured. Male B6C3F1 weanling mice were fed 10 or 25 ppm DON in AIN-76A semi-purified diet for 4 weeks then mice were euthanized by CO_2 asphyxiation prior to spleen and Peyer's patch removal. Cytokines with increased mRNA expression (fold increase over vehicle control mice, $p < 0.05$) were; TNF-α 2 fold increased at both doses in spleen but only in 10 ppm group for PP, IFN-γ 2.5 fold in 10 ppm spleen, 1.5 fold in 25 ppm spleen, IL-2 2 fold both doses for spleen and 10 ppm PP, and IL-10 3.5 fold 10 ppm spleen, 2 fold 25 ppm spleen. Since no serum levels were measured, significance of these levels of mRNA expression is unknown but animals that were gavaged with 5 mg DON/kg bw, equivalent to 25 ppm diet, for 2 days had twice the concentration of serum TNF-α although the measured mRNA was not different from controls but significantly reduced from mRNA from mice treated one day with the same dose. Either the stress of gavage two days in a row, recognized by authors, or other mechanisms resulted in modulation of the acute effect. The same DON vehicle system was used as the previous study and it was not reported if solutions

were freshly made on the second day of the trial. Sub-chronic dietary DON at 10 ppm caused significantly increased mRNA expression but lower dose responses have not been reported for the same time course. Without correlation of this data with cytokine release into the serum and from cells with the resulting biological response to any alterations observed, value of the data presented cannot be assessed.

Immunomodulation by DON in vitro

Numerous studies have investigated the *in vitro* effects of DON on immune system cells. The most relevant reports reveal information concerning alterations in cell function by direct action of DON entering the cells. Extrapolating this data to *in vivo* responses, particularly in the immune system with numerous cell and cell products expressed and regulated to elicit an appropriate response to perceived immunological threats, must be guarded.

A common approach when investigating responses of immune cells to a xenobiotic is exposure of spleen or thymus cell (whole or molecularly selected populations) suspensions to the xenobiotic in the growth media with simultaneous mitogen stimulation. Warner et al. (1994) harvested spleens and PP from B6C3F1 female mice (8 – 10 weeks old) following facility acclimation for 7 days with AIN-76A diet. Tritiated thymidine incorporation, measure of proliferation, and [^{14}C]leucine incorporation, measure of protein synthesis, were assessed in spleen cell suspensions, macrophage depleted, cultured with DON (0, 1, 10, 100, or 1000 ng/ ml media) for 72 hours. Proliferation ($p < 0.01$) and protein synthesis ($p < 0.01$) were significantly inhibited by 100 ng DON/ml in cultures stimulated with LPS but 1000 ng/ml was required to reach significant reduction with Con-A mitogen. Supernatant cytokines were measured by ELISA from CD4+ (T helper cells) that had been exposed to DON (0, 25, 100, 200 ng/ml media) and Con-A (5 μg/ml media) for 48 hours then washed and fresh media added with continued incubation for 5 additional days. IL-6 ($p < 0.01$) and IL-5 ($p < 0.05$) were significantly elevated by exposure to 25 ng/ml and 100 ng/ml DON and suppressed, not significantly, by 200 ng/ml. IL-4 was significantly elevated by 200 ng/ ml (> 10 fold, $p < 0.01$) while IL-10 was elevated but not significantly with exposure to 25 ng/ml. Concerned about washout of influence of DON in the 5 days following removal, the authors reported a similar pattern of cytokine production occurred in the same doses for continual

exposure cultures. Together this data indicated protein synthesis was inhibited in mixed cell cultures but cytokines expression was increased in a subset of splenic lymphocytes possibly indicating selective, regulatory, protein inhibition but the protein synthesis inhibition was not assessed in the T-cell subset, therefore this effect can only be hypothesized. Although the authors noted confirmation of macrophage depletion from the cell suspensions, the potential for sufficient macrophage presence to secrete activation factors for T-cell cytokine secretion could not be ruled out. Since stimulation of IL-6, IL-10 and IL-5 occurred at 25 ng DON/ ml media, it would be meaningful to conduct studies with lower doses comparable to characterize this effect in potential DON serum levels.

Ouyang et al. (1995) repeated the cytokine portion of the previous study using the same protocol (same research group) except that culture supernatant samples were taken 2 and 7 days after continuous culture of B6C3F1 murine CD4+ cells with DON (0 – 250 ng/ml) and Con-A (5 μg/ml media). The objective of the study was trichothecene comparison therefore several other cytokine profiles were reported. IL-2 was inhibited by 250 ng DON/ ml at 2 days exposure compared to untreated cells, but after 7 days incubation IL-2 was significantly increased compared to lower doses ($p < 0.05$). Interestingly the apparent IL-2 concentration, about 2.5 units/ml, at 250 ng DON/ml media did not change over the time periods but the controls and lower doses had initially higher levels of IL-2, about 5 U/ml, then declined to about a third, 0.5 U/ml. IL-5 and IL-4 were inhibited in a dose responsive manner by ≥ 100 ng/ml (2 days) but was elevated by100 ng/ml and almost not detected in the 250 ng/ml (7 days). This pattern of inhibition at 2 days and stimulation at 7 days was also evident with equivalent concentrations for NIV but 2 – 3 X higher concentration was required for 15-ADON and 10 X or more for 3-ADON. Cytokine mRNA expression was measured at 2 days DON exposure with significant induction at 50-100 ng/ml for IL-2 and 50 ng/ml for IL-4. IL-5 and IL-6 mRNA were not affected by the treatments. The results of this study indicated the importance of considering acute versus chronic DON exposure effects. Initial contact with the mycotoxin inhibited cytokine production at higher doses than were required for increased synthesis, or lack of degradation, at lower doses with sub-acute exposures, keeping in mind the results are for an isolated lymphocyte cell type.

Miller and Atkinson (1986) investigated low dose responses of peripheral blood lymphocytes from PVG rats, no sex stated, to DON in media. Lymphocytes, separated by Ficoll-Hypaque gradient, were incubated with PHA and DON (0 – 200 ng/ ml media, five replicate wells per concentration) for 42 hours then [^{3}H]thymidine (1 μCi/ well) was added until completion of incubation at 48 hours. Stimulated proliferation, about 130% controls, occurred for DON concentrations between 0.005 and 0.5 ng DON/ ml media (no statistics) and was inhibited by 50 or 100 ng/ml. Release of mitogenic substances from cell subsets was investigated by isolating peritoneal macrophages from male PVG rats and incubated, after removal of nonadherent cells, with DON (150 or 300 ng/ml media), Con-A (mitogenic) or cycloheximide, prototypic protein synthesis inhibitor. The media was removed after 24 hours and fresh media was added prior to additional 16-20 hour incubation. Media was removed and used as a conditioning media added to peripheral rat lymphocytes, described previously, for assessment of proliferation. Conditioning media, supernatant from macrophages exposed to 300 ng/ml DON, caused significantly increased lymphocyte proliferation ($p < 0.001$) compared to Con-A-conditioning media and this media was also found to stimulate significant proliferation in the thymocyte co-stimulator assay, C3H murine thymocytes stimulated with PHA, compared to Con-A treated macrophage conditioning media or control media, indicating considerable IL-1 activity. The author concluded that DON stimulated mitogenic cytokine release from macrophages contributing to lymphocyte proliferation but this was contradictory to the concentrations reported, as the stimulatory effect on incubating DON directly with lymphocytes was markedly lower than that used to stimulate cytokine release in macrophages. IL-1 was the cytokine reportedly responsible for this effect but IL-12 had not yet been described in the literature and could contribute to this effect. This report was the only one known to investigate these extremely low DON concentrations on peripheral lymphocytes of any species.

Peritoneal macrophages from female Swiss mice were exposed to DON (1 – 1000 ng/ ml media) for 4 hours then assessed for alteration of microbiocidal and phagocytic activity (*Saccharomyces cerevisiae* challenge), super oxide production, phagosome-lysosome fusion and viability (Ayral et al., 1992). While macrophage viability was not affected (LDH release) across all DON concentrations, microbiocidal and phagocytic activity was significantly

reduced by ≥1 ng/ml ($p < 0.05$) with reduced super oxide production compared to controls. Phagosome-lysosome fusion was inhibited by ≥100 ng DON/ml media. This experiment was replicated five times with duplication each time therefore the data appears valid. Clearance of the challenge organism required opsonization and apparently from this data, lethal oxidative mechanisms once engulfed. The low threshold sensitivity of murine peritoneal macrophages may explain previously described data for *in vivo* inhibition of *M. avium* clearance in mice as this organism requires intracellular growth. The authors do not speculate further the mechanism of action and there have not been any reports investigating *ex vivo* responses of peritoneal macrophages to *in vivo* DON exposure.

Numerous studies have been published investigating *in vitro* exposure of human peripheral lymphocytes to DON in media. Meky et al. (2001) separated lymphocytes (healthy blood donors) from peripheral blood by Ficoll-Hypaque gradient centrifugation and exposed them to PHA and DON (50 – 500 ng/ml) for 5 days prior to cytotoxicity assessment with MTT assay. Cytokines were also assayed (ELISA) in supernatants (harvested daily) from lymphocytes exposed to DON (0, 100, 200, or 400 ng/ml media) ± PHA for 3 days or 8-9 days. DON caused reduced lymphocyte proliferation in concentrations greater than 100 ng/ml and an IC_{50} 216 ng/ml was determined from the standard curve. The authors indicated reduced proliferation without reduced cell viability but did not report the basis for this statement. IL-2, but not IL-4 or IL-6, was stimulated in culture after 72 hours at 200 and 400 ng/ml compared to cells exposed to PHA alone ($p < 0.05$). When cultures periods were extended (DON 400 ng/ml) IL-2 was significantly elevated at 24 hours and increased every 24 hours with peak production at 113 hours ($p < 0.05$). IL-4 was significantly elevated at 72 and 113 hours of incubation ($p < 0.05$) but then declined. IL-6 was depressed at 24 hours incubation ($p < 0.05$) then was below but not significantly different from controls. When two donors' lymphocytes were compared over 9 days of incubation with DON (200 ng/ml), IL-2 peaked at 72 hours for both donors, returning to control levels for one donor at 120 hours and 168 for the other. IFN-γ was inhibited for 72 hours for both donors then reached peak production at 120 hours for one donor and 168 hours for the other with no decline in concentrations for the remainder of the culture period (216 hours). IL-6 was unchanged by DON in either donor. An omission in this study was historical information about the donors.

Confounding immunomodulatory influences such as smoking, prior mycotoxin intake, and nutritional status were not reported. These factors were commonly under-reported in studies of this type. A second concern was the length of incubation without mention of media supplementation or change. Potentially cells could become stressed due to nutrient utilization over 8-9 days in culture. Lower concentrations of DON (biologically relevant) in culture would provide data that could more accurately be compared to rodent studies. Although human serum levels of DON have not been reported, it does not seem feasible that the upper levels tested in this study would occur with common food levels.

A more comprehensive approach to *in vitro* exposure of human peripheral blood lymphocyte to DON was reported by Berek et al. (2001). Lymphocyte proliferation was assessed, [^{3}H]thymidine incorporation, in PHA and Con-A mitogen stimulated cultures exposed to 10 – 5000 ng DON/ml. Lymphocytes, void of adherent cells, were incubated with ^{51}Cr labeled Rh+ Type O RBCs, and anti-Rh(D) antisera, 1:20 vol/vol dilution in media, along with different concentrations of DON to assess antibody-dependent cellular cytotoxicity (ADCC). NK cell cytotoxicity was evaluated by incubating peripheral lymphocytes with ^{51}Cr labeled K-562 cells and different concentrations of DON for 16 hours prior to supernatant collection to count radioactivity release. Three donors were tested and the authors indicated undetectable DON levels in sera by ELISA. The complete data sets were not reported but lymphocyte proliferation was inhibited ($p < 0.05$) by 100 ng DON/ml media with both mitogens. Both cytotoxicity assays were significantly inhibited by 50 ng DON/ ml ($p < 0.05$) although it did not appear to be dose dependent for ADCC as 22 ± 5% inhibition was reported for 50, 100, and 1000 ng/ml. There were some serious problems with this report as graph and figure captions did not match the data represented making the results difficult to accurately identify. A common omission in these studies was not reporting results for all concentrations tested and since most study objectives were immunosuppression, any effects observed at lower doses were ignored. These authors also stated concentrations for this study were selected based on concentrations that can occur in 'normal' human peripheral blood but no reference or rationale was given for this statement. This study was included in this review because it was the only report of cytotoxicity assessment in human lymphocytes.

Sensitivity of these cells to DON was not indicated although these would be considered acute exposures as the cells were only exposed to the toxin for 16 to 18 hours.

Immature cells in the bone marrow or thymus are exposed to tissue levels of DON when ingested at high enough concentrations in most monogastric animals as reviewed earlier. A series of experiments were conducted to investigate the relative sensitivities of progenitor cells of different cell types. Lautraite et al. (1997) investigated the sensitivity of rat bone marrow cells and human umbilical cord progenitor cells. Cells from both species were incubated with conditioning media specific for activation of granulocyte-monocyte progenitors and DON at different concentration on solid media for 14 days, each concentration in triplicate, each experiment repeated three times. These cells form colonies (more than 50 cells), macroclusters (20 to 50 cells), and microclusters (5 to 20 cells) in culture and these were scored microscopically at 7, 10 and 14 days of culture. Human cell growth, % of control total aggregates of cells, was inhibited to some degree by all concentrations tested (as low as 2.96 ng DON/ml) with IC_{50} values of 8.9 ng DON/ml at 7 days, 8.6 ng/ml at 10 days, and 11.5 ng/ml at 14 days incubation. Human cells were destroyed by $\geq$ 74 ng/ml. Rat bone marrow cells were significantly ($p < 0.05$) less sensitive. All rat cells were destroyed by 296 ng DON/ml and IC_{50} values for 7, 10 and 14 days of incubation were 77, 44.4, and 47.4 ng DON/ml respectively. Cell formation type was affected as concentrations that inhibited overall growth had higher frequency of microclusters compared to untreated controls whereas at lower concentration the distribution was even between colonies, macro- and microclusters. The authors emphasized that this assay was the observation of cells produced from several divisions from the original progenitor cells, thus if differentiation is disrupted growth may be arrested. This was suggested in the response of granulocyte-monocyte progenitors due to increased frequency of microclusters indicating arrest of mitosis earlier in differentiation than larger aggregates of cells. There was also differences in culture time for different cell progenitors to acquire cluster formation so that assessing growth patterns at different time points during exposure indicated sensitivity of late-aggregate-forming cells although the specific cell type was not proposed. The importance of this report was the low level sensitivity expressed by human granulocyte-monocyte progenitors. Since the umbilical cord samples were obtained randomly

and without contact with the donor, prior exposure to mycotoxins was not reported to be assessed by the investigators.

Froquet et al. (2001) investigated the response of human megakaryocyte (platelet) progenitors. A similar system was used as the previous study except the umbilical cord cells were separated by gradient density (d = 1.077 g/ ml) centrifugation to acquire light density cells. DON in acetone was added to the cells in semisolid media along with human recombinant thrombopoetin (50 ng/ml), human recombinant IL-6 (10 ng/ml) and IL-3 (10 ng/ml) as growth stimulation factors. Following 12 days of incubation, cells were fixed and immunocytochemically stained for CD 41, primary monoclonal antibody to a glycoprotein surface marker on megakaryocytes. Colony formation scoring was conducted as previously described. DON at 74 ng/ml media was not cytotoxic although total colony growth was 55 ± 20% of untreated controls. Significant inhibition of colony formation ($p < 0.001$) and stimulation of microclusters ($p < 0.05$) also occurred at this dose. Lower doses, 22.2, 7.4 and 2.9 ng DON/ ml, did not alter colony formation. These cells were not as sensitive to DON exposure under optimal growth condition as white blood cell progenitors tested previously but the sensitivity was within the suspected range of *in vivo* serum levels.

Froquet et al. (2003) investigated the effects of DON on mature human peripheral RBC, WBC and platelets. Platelets were separated from blood donated by healthy human adults by centrifugation and added back to plasma prior to exposure to DON in the plasma. Coagulation (measured by prothrombin time, activated partial thromboplastin time, and thrombin time assays) and platelet aggregation (response to collagen or adenosine-di-phosphate) was not affected by concentrations as high as 2.96 µg DON/ml plasma. Red blood cells and WBC were separated from umbilical cord blood by gradient density centrifugation (d = 1.077 g/ml). RBC were washed twice in glucose medium to retard spontaneous hemolysis and normalized to 20% Hct. WBC were washed and mixed with plasma. Both cell type suspensions were incubated in solution for 24 hours at 37°C with different concentrations of DON prior to viability assessments. WBC were not affected by DON up to 2.96 µg/ ml plasma after 6 hours incubation but at this concentration after 24 hours viability was decreased 50% ($p < 0.05$) of untreated controls based on hemocytometer counting, assuming Trypan blue dye exclusion but not stated. RBC hemolysis was not

affected by any concentration although D-glucose concentration (0.2 ± 0.8 vs. 0.6 ± 0.8 μMol/ml) and L(+)lactate concentration were decreased (4.5 ± 2.9 vs. 4.8 ± 2.4 μMol/ml) by 2.96 μg DON/ ml at 24 hours compared to solvent control incubations. Glutathione (reduced form) was also reduced compared to solvent controls (4.6 ± 1.6 vs. 5.5 ± 3.0 μMol/ g Hb) following 24 hour exposure to 2.96 μg DON/ ml. Although the majority of data reported here was negative, this indicated relative tolerance of mature circulating RBC, WBC and platelets to short term exposure of DON. Since the lifespan of RBC is 120 days, investigation of effects due to longer exposures are warranted although culture conditions may be difficult. Together with data from the previous two reports, effects observed *in vivo* were more likely due to progenitor cell sensitivity rather than effects on mature circulating cells.

Immune function alterations following DON exposure in mice have included; reduced antibody titers to sRBC (Tryphonas et al., 1984), altered serum protein profiles (Tryphonas et al., 1984, 1986), thymic atrophy (Robbana-Barnat et al., 1988), reduced clearance (Pestka et al., 1987) and reduced time of death (Tryphonas et al., 1986) to *L. monocytogenesis* challenge, reduced clearance of *S. enteritidis* (Hara-Kudo et al., 1996), and lack of defense against mycobacterial mastitis (Atroshi et al., 1994). Acute and sub-chronic doses have stimulated cytokine mRNA expression with increased serum cytokines (Zhou et al., 1997, 1998). *In vivo* immunologic studies have not been conducted in species other than mice but *in vitro* investigations have been conducted in several species including humans. Rodent spleen cells exhibit dose-response effects with stimulated proliferation at very low doses (Miller and Atkinson, 1986) and inhibition at higher biologically relevant exposures (Miller and Atkinson, 1986; Warner et al., 1994). Cytokine release from murine spleen cells (Ouyang et al., 1995) and human peripheral blood lymphocytes (Meky et al., 2001) exhibited time and dose dependent response with inhibition at 2 days culture but stimulation after longer periods. DON induced the release of lymphocyte growth factors from mouse peritoneal macrophages (Miller and Atkinson, 1986) and functionality of these cells was inhibited by extremely low doses *in vitro* without any effect on viability (Ayral et al., 1992). Human peripheral blood NK cytotoxicity and lymphocyte proliferation were inhibited (Berek et al., 2001). Progenitors of granulocytes (Lautraite et al., 1997) and megakaryocytes

(Froquet et al., 2001) exhibited cytotoxicity to low DON doses but mature RBC, WBC, and platelets were not affected by acute exposure (Froquet et al., 2003).

Overall conclusions regarding immunomodulation by DON

Immunomodulation in response to DON exposure does not appear to be species specific although sensitivity due to bioavailability *in vivo* may affect levels in contact with immune cells. While *in vitro* studies help define explicit responses, these can only reflect direct responses by the specific cells exposed, and do not include the influences of the whole animal. DON has been shown to influence cytokine mRNA expression and cytokine secretion *in vivo* and *in vitro* and most likely is an important mechanism of biological influence. Effects were dependent on dose and length of exposure with particular sensitivity observed in some cell types (macrophages and stem cells). Although functional assays have been conducted on human cells *in vitro*, little is gained from this work without epidemiologic data documenting extensive dietary and xenobiotic histories, actual plasma and urine mycotoxin levels, and conducting immunologic assays to determine basal function as well as host resistance. Anecdotal evidence exists for immunosuppression in humans (Bhat et al., 1989) but experimental evidence is lacking.

Acute Exercise Stress Effects on the Immune System

Acute stress has been induced in mice by numerous techniques including electric shock, rotational stress, swimming, and restraint. The focus of this review will be on the main aspects of acute exercise stress and the effects induced in the immune system. We were interested in this model because segments of the population are exposed similarly, military personnel in particular, and this may serve as a model for other acute stressors perhaps psychological. Some relevant human studies on this subject will be introduced to correlate the appropriateness of the mouse model. Treadmill running to exhaustion was our model for acute exercise stress. Hoffman-Goetz et al. (1988) investigated the effects of acute exercise to fatigue and 8 week exercise training on splenic immune functions, only the information directly related to acute exercise stress will be reviewed. Male mice (C57BL/6J) were group housed and acclimated to the facility for at least a week prior to starting the training program or waiting until the end of the study to participate in one time exercise to fatigue on a

treadmill. Exhaustion was determined when the mice could no longer maintain speed with physical prodding (60 – 70 minutes). Mice were rested for 30 minutes before termination. Plasma corticosterone was determined by extraction and duplicate samples compared spectrophotometrically with a standard curve. Single cell spleen suspensions were prepared, cell viability determined by Trypan blue dye exclusion, enumeration by hemocytometer, and lymphocyte proliferation assays (I X 10^6 cells/ well in triplicate) measured by [^{3}H]thymidine incorporation following media only, LPS (1.0 μg/ml), and pokeweed mitogen (PWM) (0.5 μg/ml) cellular incubations. Plasma corticosterone levels were significantly higher in mice that had been exercised to exhaustion ($p < 0.0001$) compared to unexercised controls. There were no significant effects on total spleen cell numbers or response of the spleen cells to either mitogen. The authors concluded that corticosterone elevation was not responsible for splenic immunosuppression as hypothesized. This study performed a limited number of assays but with adequate sample size ($n = 10$) and statistical analysis to support their conclusions.

Further assessment of the effects of acute exercise stress using the treadmill model was continued by Hoffman-Goetz et al. (1989). Male mice (C3He) were treated similarly to the prior study. Following 30 minutes rest after exhaustion, mice were terminated and spleen, thymus, and several lymph nodes collected. Cell populations within each tissue were determined by monoclonal antibody labeling of cells for expression markers Thy1.2 (all T-lymphocytes), L3T4 (T-helper cells), Lyt2 (T-suppressor/cytotoxic cells) and Ig expression (B-cells) with enumeration by direct immunofluorescent flow cytometry (20,000 cells counted per sample). Consistent with the previous study, there were no differences between treatment groups for total cell concentration in these three tissues. Thymus cell composition was altered by acute exercise stress resulting in suppression of Thy1.2+ cells ($p < 0.05$) and Lyt2+ cells ($p < 0.001$) in response to this treatment. Splenic Ig+ cells were significantly reduced ($p < 0.008$) in acutely exercised mice compared to sedentary controls but other cell types were not affected. No differential effects were observed in the lymph nodes. Together with the previous study, assuming corticosterone elevation in these mice, splenic cellular population was not altered by this stress other than reduced Ig+ B-cells. Differences observed between lymphoid organs may suggest alteration of local environments within these organs

resulting in marker loss or trafficking these cells into the circulation. It is important to note that the exercise stress occurred over about an hour followed by a thirty minute rest period thus cellular responses observed occur rapidly.

Further assessment of differential effects on lymphocyte subsets due to acute exercise stress was conducted by Randall Simpson et al. (1989). The same mouse strain and exercise protocol ($n = 6$) as above was used including labeling and flow cytometry of the cells. Prior to labeling cell subsets, spleen cell suspensions (10^6 cells per 500 µl per well in quadruplicate) were incubated with media alone, Con-A (1.0 µg/ml) or Con-A plus purified IL-1 (1 LAF unit) for 96 hours. In response to Con-A, a higher percentage of Thy1.2+ (pan T-lymphocytes) ($p = 0.0034$) as a result of a significant increase of Lyt-2+ (T-suppressor/cytotoxic) cells ($p < 0.001$) compared to sedentary controls. There was a trend of reduced Ig+ cells ($p = 0.06$) by acute exercise stress in response to mitogen activation. Addition of IL-1 to the culture was not different from Con-A for any of the cell subsets. Increasing the number of T-suppressor cells upon activation following a bout of acute exercise stress could be a mechanism to control mitogenesis but as the authors discuss, other suppressor cells are also activated during mitogenic stimulation in culture (macrophages), T-suppressor cells can provide helper functions or other suppressive molecules produced in culture (cytokines?). These results confirm the results of the previous study indicating no differences in un-activated spleen T-cell populations but distinct responses upon activation occur. Ig+ B-cells were suppressed by the stress with or without mitogenic activation. Further investigation will be required to investigate the mechanisms of these effects and the *in vivo* consequences, if any. As previously stated, *in vitro* studies limit the extrapolation to *in vivo* effects due to influences from many tissues and systems particularly in the immune system with complicated and overlapping regulation.

The effects of treadmill exercise to exhaustion on NK cytotoxicity was assessed (Randall Simpson and Hoffman-Goetz, 1990). The same mouse strain and exercise protocol was used as the previous three reports. The classic ^{51}Cr release assay was conducted with spleen cell suspension (1.5×10^7 cell/ml) and YAC-1 cells (1×10^5 cell/ml) mixed in effector: target ratios 150:1, 75:1, 37.5:1, and 18.25:1 in triplicate and incubated 4 hours prior to assessment of radioactivity in supernatants. NK enumeration in the spleen cell

suspensions was done by labeling cells with asialo GM_1 ($ASGM_1$, NK marker) and detecting indirect immunofluorescence by flow cytometry. Splenic tissue was analyzed for catacholamines by extraction and HPLC. No treatment effects were detected for norepinephrine levels in the spleen but epinephrine levels were significantly higher in mice exercised to fatigue (33 ± 3.0 pg/mg protein, $p < 0.05$, $n = 8$) compared to sedentary controls (10.9 ± 1.3 pg/mg protein, $n = 9$). NK cytotoxicity was significantly reduced by acute exercise stress ($p < 0.05$) compared to sedentary controls at 150:1 E:T ratio and the same pattern was observed across all ratios. NK cells expressing $ASGM_1$ was also reduced by exercise treatment ($p < 0.05$). The authors reported similar inhibition of NK cell cytotoxicity observed with other acute stressors, but only transiently. This series of experiments reported alterations in spleen and thymus lymphocyte populations and functionality with elevations in corticosterone and epinephrine due to acute exercise stress. Information on alterations of cytokine production or receptor expression for this model of acute stress has not been reported.

Mechanisms of cellular immune effects from acute exercise stress were investigated by Azenabor and Hoffman-Goetz (1999). Female C57BL/6 mice exercised on a treadmill to exhaustion and killed immediately or rested for 24 hours were compared to sedentary controls ($n = 10$) for splenic and thymic cellular concentration of antioxidant enzymes superoxide dismutase (SOD) and catalase. Lipid peroxide was assayed from cell-fragment pellets and plasma uric acid and ascorbic acid was measured. The thymus had a significantly higher concentration of lipid peroxidase ($p < 0.05$) immediately after acute exercise and remained high 24 hours later compared to sedentary controls. The spleen also had higher lipid peroxidase ($p < 0.05$) due to acute exercise and it continued to climb 24 hours later although the thymic levels were 3-4 times higher across all treatment groups. Both antioxidant enzymes in both tissue types were significantly ($p < 0.05$) consumed immediately following acute exercise but returned to control levels 24 hours later. Plasma uric acid was reduced but not significantly by acute exercise stress at both times but plasma ascorbic acid was significantly reduced only after 24 hours post-exercise ($p < 0.05$). This pattern of responses reflected production of oxidative damage and built-in mechanisms of compensation as discussed by the authors. Under these controlled conditions with only one

stressor, this response seemed appropriate but the addition of other stressors such as nutritional deficiencies or infectious agents may overwhelm the compensatory capacity of the animal.

The observation of elevated corticosteroids following stress has been commonly documented. The effect of this hormone response on the immune system has been studied for a long time particularly since this class of compounds is used as treatments for allergies, asthma, and immune cell cancers (lymphoma). Specific immune responses of mice to the acute stress following adrenalectomy provide evidence for influence of corticosterone (Esterling and Rabin, 1987). Male Swiss-Webster mice were either adrenalectomized under anesthesia or under went a sham operation then both groups were allowed to heal to 2 weeks. All mice were injected i.p. with sRBC (0.4 ml 5% in PBS) and half of each group were stressed by rotation (5 X 60 minute cycles, 10 minutes rotating 100 rpm followed by 50 min rest) immediately after the sRBC priming and for 3 additional days. The other half of the surgical groups rested for 24 hours after sRBC priming then was rotationally stressed for 3 days. Immunized unstressed controls were maintained and killed in the same timeframe (n = 7 – 13 mice per group). Antibody titers to sRBC (microhemagglutination) were measured in blood collected at time of death. Single cell spleen suspensions were prepared and the hemolytic plaque assay and immunofluorescence staining for enumeration of lymphocyte subsets was conducted. There was a significant effect of stress on the PFC ($p < 0.001$) as the animals stressed immediately after sRBC priming were not significantly different from primed but not stressed controls but the animals given 24 hours rest had significantly reduced PFC regardless of adrenal presence. The same pattern occurred for serum antibody titers ($p < 0.01$). T-cell populations were adrenal dependent but did not respond to timing of stress. Both T-helper (Ly1+) and T-suppressor (Ly2+) cells were significantly higher in adrenalectomized mice ($p < 0.05$) if stress occurred immediately after sRBC priming but T-suppressor cells were not different from non-stressed controls for either adrenal treatment. The authors were careful to express limitations of interpreting and extrapolating this data to general conclusions about the relationship between stress, adrenal hormones, and immune responses because timing, frequency, severity, and duration of stress must be considered to compare between studies. The finding of an adrenal independent effect on suppression of

PFC and antibody titers to sRBC in the animals stressed 24 hours after immunization may indicate a sensitivity of a particular phase of cell activation in the process of epitope recognition or protein processing. This study has limited relevance to our primary research but it did indicate that corticosteroids are not the only endocrine product of acute stress that can affect antibody production and that interactions occur between times of immunization and stress that can significantly affect the resulting immune response.

The use of mouse models to study the effects of acute exercise stress on immune function has been utilized because responses have been comparable to humans and relatively large studies can be conducted in a controlled environment. Humans can be studied directly to some extent. Weinstock et al. (1997) investigated the release of cytokines into the urine and serum and from whole blood cell cultures of male athletes before and after exhaustive exercise stress (sprint triathlon, average completion time 68 minutes). Blood and urine was collected 24 hours prior to exercise, 1 and 20 hours after exercise. Cytokines were measured in serum and urine by ELISA. Whole blood was cultured with LPS (1 ng/ml), PHA (5 μg/ml), Con-A (10 μg/ml) or culture media only for 24 hours then supernatant was removed after centrifugation. Cell counts performed on whole blood revealed a significant increase ($p < 0.01$) 1 hour after exercise in total WBC, granulocytes (neutrophils) and monocytes while the lymphocytes were decreased ($p < 0.01$) compared to 24 hours before exercise. Cell counts had returned to pre-exercise levels 20 hours after exercise. Immediate exercise dependent elevations in IL-6 ($p < 0.01$) and TNF-α ($p < 0.05$) was observed in all subjects. Although several subjects expressed them, IL-2 and IFN-γ did not exhibit exercise dependent changes. Only IL-6 had significantly higher frequency of detection in urine 1 hour after exercise (13/15 subjects, $p < 0.01$) compared to pre-exercise (4/15) or 20 hours post-exercise (2/15). Spontaneous release of cytokines from whole blood cultures indicated suppression of IL-6 and TNF-α at both time points post-exercise, IL-1β was suppressed only at 20 hours post-exercise while IFN-γ was elevated by 1 hour post exercise and remained higher compared to pre-exercise levels. IL-2 was not detected in media only cultures. LPS (B-cell) mitogen stimulated cultures significantly depressed TNF-α, IL-1β, and IFN-γ while stimulating IL-6 at 1 hour post-exercise. Con-A depressed IFN-γ 1 hour post-exercise and stimulated IL-2 release 20 hours post-exercise. PHA depressed IL-2 at 1 hour post-exercise but slightly

stimulated its secretion 20 hours post-exercise compared to pre-exercise levels. Since whole blood was not corrected for elevations of cell counts 1 hour post-exercise, cytokine concentrations were normalized to concentration per 1000 lymphocytes or monocyte, depending on assumed expressing cell type, resulting in only IL-6 significantly depressed (concentration per 1000 monocytes) in LPS stimulated cultures 1 hour post-exercise. This study was significant in that it showed the transient nature of immune cell and cytokine production following acute exercise stress. It was a good study to investigate human *in vivo* effects because the lymphocytes were kept in the blood matrix so that cells could influence each other by the cytokines produced. Stress hormones present in the blood were minimally diluted thus potentially continuing to exert effects over the 24 hour incubation period. These men were trained so the results reported here could not be extrapolated to untrained individuals but the responses were pair-wise compared to themselves so appropriate statistical analysis was conducted. The training regimes of the subjects were quite varied and may also affect results although in the previous mouse studies, training (results not included) was independent of the acute response. Other than differential WBC count, there was no information to determine the lymphocyte cell types as had been done in the previous mouse studies.

Acute exercise stress has a unique profile of responses in the immune system that can be modified depending on the intensity, duration, and type of exercise conducted. There were apparently complicated neuroendocrine effects directly on circulating and tissue based immune cells involving in particular corticosteroids and catacholamines. Numerous murine models of acute exercise stress to study the mechanisms have been developed but the treadmill model was chosen due to access to equipment and prior experience. Although murine cytokine data did not appear to be available for acute exercise stress in an appropriate model, some human data was available that appeared comparable to the murine model. Mice forced to exercise to fatigue on a treadmill and rested for 30 minutes exhibit: elevated plasma corticosterone levels without altering splenocyte proliferative responses to LPS or pokeweed mitogen (Hoffman-Goetz et al., 1988); decreased Ig+ spleen cells, decreased total T-lymphocytes with specific reduction of T-suppressor cells in the thymus but no alterations in the lymph nodes (Hoffman-Goetz et al., 1989); all T-lymphocytes, T-suppressor/cytotoxic

and Ig+ spleen cells stimulated significantly by Con-A (but not additionally by IL-1) compared to un-stressed control mice (Randall Simpson et al., 1989); NK cytotoxicity and NK spleen cells significantly reduced while splenic epinephrine was elevated (Randall Simpson and Hoffman-Goetz, 1990). Thymus and spleen undergo oxidative stress following acute exercise (Azenabor and Hoffman-Goetz, 1999). Antigen specific PFC is suppressed if acute stress occurs 24 hours after immunization but not immediately and independent of corticosterone where as suppression of lymphocyte subsets did appear to be corticoid dependent (Esterling and Rabin, 1987). Men acutely exercise-stressed had elevated blood WBC, neutrophils, and monocytes with concurrent lymphocytosis (Weinstock et al., 1997). These men also secreted increased levels of IL-6 into blood and urine and whole blood cultures revealed suppression of IL-6 and TNF-α and stimulation of IFN-γ spontaneously released, while mitogen activation in these cultures tended to suppress cytokine release from blood collected 1 hour post-exercise compared pre-exercise levels (Weinstock et al., 1997). The body of literature concerning exercise and immunity is immense. The murine model appears comparable to human responses although numerous authors caution comparing across exercise models.

Interaction of DON and acute exercise stress?

It seems reasonable that dietary DON exposure and acute exercise (or other) stress could occur in the same person, or group. Dietary animal studies have indicated toxic endpoints in monogastric animals at levels of exposure frequently occurring in worldwide food sources. Both dietary DON and acute exercise stress have caused immunosuppression in mice. The mechanisms of immune function alteration following acute exercise have been relatively well characterized with oxidative stress playing a significant role. Although mechanism of toxicity for DON exposure was characterized early, evidence is building for mechanisms involving cellular signal transduction pathway activation or inhibition. Oxidative stress also affects these pathways which could be a mechanism for interaction between DON and acute stress. No published reports have investigated similar interactions.

CHAPTER 3. CAFFEINE AS INTERNAL STANDARD FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF DEOXYNIVALENOL IN WHEAT SAMPLES

A paper to be submitted to *Journal of AOAC INTERNATIONAL*

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Abstract

A method was developed to utilize caffeine as an internal standard for analytical detection of deoxynivalenol (DON) in wheat samples using high performance liquid chromatography (HPLC) with ultraviolet detection. Ground wheat samples were extracted with aqueous acetonitrile and spiked with 30 μg caffeine (CAF). Sample aliquots were cleaned up through an alumina-charcoal column and analytes were separated with linear aqueous methanol gradient, UV detection at 220 nm. Retention times for DON and CAF were approximately 9.0 min and 16.0 minutes respectively. Fungal culture material (124 μg/g) was added to commercial wheat flour to achieve mean DON concentrations of 0.26, 0.51, 1.99, and 5.96 μg/g. Three replicates of each concentration were analyzed daily and repeated four days. An internal standard curve was developed plotting sample concentration against area response ratios (DON/CAF). Correlation coefficient (r) was 0.983 and no significant difference between or among days of analysis occurred. Recovery of DON spiked samples at 1.00 μg/g was 100.5% in this system. This method will allow analysis of samples using an accessible, stable, and inexpensive compound not likely to be found in most samples of interest.

Introduction

Deoxynivalenol (DON) (Figure 3.1) is a secondary metabolite of *Fusarium sp* that occurs commonly worldwide in staple foods (Placinta et al., 1999). Methods for detection of DON include TLC, GC, ELISA, and HPLC (Krska et al., 2001). Most methods specify

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standard addition spiking as confirmation of DON presence. Due to the FDA advisory level of 1 µg/g in food for human consumption, efforts have not been made to reduce the limit of detection below 0.5 µg/g. Internal standard addition has not been published for HPLC/UV analysis. Caffeine (CAF) (Figure 3.1) was chosen as a candidate for an internal standard due to an optimum UV absorbance wavelength of 210 nm, low molecular weight of 194, compatible solvent solubility, and chemical stability in DON analytical methods (Merck, 1996). It also qualified as an internal standard due to absence in samples of interest. The purpose of this study was to develop a "gold standard" method in our laboratory for comparison to future screening method research.

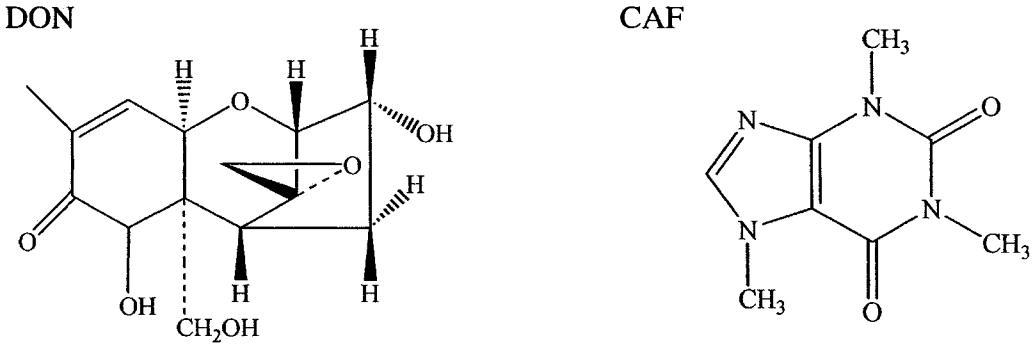


Figure 3.1. Molecular Structure of Deoxynivalenol (DON) and Caffeine (CAF).

Materials and Methods

Chemicals

DON, CAF, yeast extract, potato dextrose agar (PDA), and carboxymethyl cellulose (C-0806) (CMC) were from Sigma (St. Louis, MO). HPLC grade acetonitrile and methanol were from Fisher Scientific (Pittsburgh PA). Water was filtered though Waters Milli-Q® system and buffered to pH 7.0 with 3.0 M NaOH (if needed) prior to use. Ground charcoal was from Acros Organics, NJ and was from a single lot. Fusarium cultures were purchased from Fusarium Research Center, Pennsylvania State University, University Park, PA.

Samples

Negative wheat flour samples were purchased from local commercial sources. DON contaminated culture material was produced (Witt et al., 1985) on autoclaved rice using

lyophilized *Fusarium graminearum* (R-5245, USA OH). Culture material was dried, ground to a meal, and stored at –20°C. Analysis by HPLC determined DON concentration by standard curve. The culture material used for internal standard curve development contained 124.18 µg DON/g dry weight.

Extraction and Cleanup

Fungal culture material was added to 25 g wheat flour to achieve target DON concentrations of 0.25, 0.50, 2.0, and 6.0 µg/g. Acetonitrile and water (84:16) was added (100 ml) to each sample along with 30 µg of caffeine dissolved in extraction solvent. The sample was blended at high speed in ½ pint blender jars for 3 minutes, filtered through paper coffee filters into flasks and covered with Parafilm. Solid phase extraction columns were constructed of 1 gram alumina (80-200 mesh) 40:1 with ground charcoal in a 5 ml syringe barrel with a fitted Whatman #4 filter paper frit at the top and bottom of the packing. Five ml sample extract was applied to the column and allowed to flow by gravity into a 15 ml glass test tube. Two ml of the eluate was quantitatively transferred to a 12 X 75 mm glass cuvette and dried under a gentle stream of nitrogen in 50° C water bath. The residue was dissolved in 400 µl of 15% methanol: buffered water and filtered through 0.45 µm 4mm PVDF syringe filter into HPLC autosampler vial.

HPLC Analysis

The HPLC system was a Hewlett Packard 1050 with UV diode array detector, autosampler, and degasser, with an YMC-Pack ODS-A C18 RP, S5µ 4.6 X 100 mm, with 4.6 mm guard column from Waters (Franklin, MA). Data acquisition and analysis was performed by ChemStation software. Injection volume was 50 µl with 0.7 ml/min flow rate by linear gradient of 15-20% methanol over 25 minutes with a step-up to 80% methanol for 5 minutes for wash out and re-equilibration at 15% methanol for 15 minutes. Total run time was 50 min/sample. DON standard curve was determined over 0.2 to 6.0 µg/g sample and standards were run each day of sample analysis. For internal standard curve validation, three replicates of each concentration were prepared daily and repeated on four separate days. Statistical analysis was performed with SAS (Cary, NC) using weighted least squares linear regression.

Safety

Culture material was handled in a certified BL2 laminar hood. Biohazard safety and hazardous waste generation requirements of Iowa State University Environmental Health and Safety were followed. A USDA permit was obtained for the transport and use of Fusarium cultures.

Results and Discussion

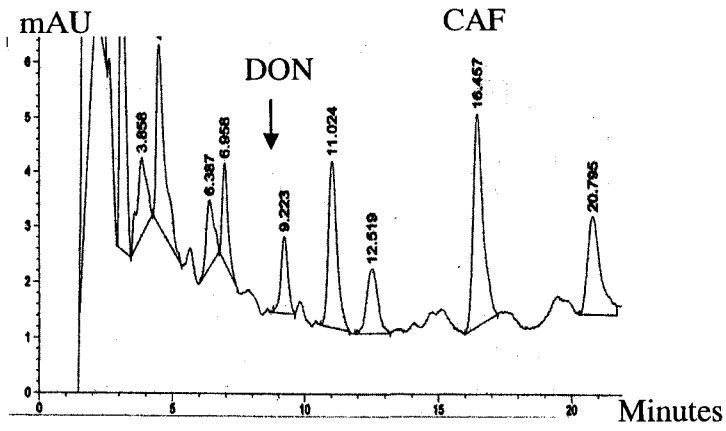
Extraction and Cleanup

Several processes in this method required alteration from a peer-reviewed method (Trucksess et al., 1998). The pH of laboratory filtered Milli-Q® water tended to be acidic causing a source of variability in CAF recovery. pH was monitored for all water used in sample processes and adjusted to 7.0 ± 0.2 if necessary. The commonly used commercial SPE column for trichothecene mycotoxins (Mycosep™, Romer Labs, Inc., Washington MO) resulted in excessive loss of caffeine. Our 40:1 alumina-charcoal column produced a cleaner chromatograph and greater DON recovery as compared to the commercial SPE column. Numerous ratios of alumina, charcoal and Celite were tried, as well as cation exchange resin and Sep-Pac C-18 columns. Alumina and charcoal SPE columns could be made ahead of time and stored at room temperature covered with foil. No vacuum apparatus was required for extract flow through the column at 1 ml/minute and the eluate was clear and colorless.

LC Analysis

DON standard curve was prepared from analysis of control solutions in concentrations of 0.2, 0.4, 1.0, 2.0, and 6.0 µg/g. Linearity was verified daily across concentration range with an average correlation coefficient (r) of 0.999. Limit of detection was 5.8 ng/injection based on signal to noise (3:1) calculation (Cunico et al., 1998). Limit of quantification was 0.4 µg/g based on multiple injections yielding relative standard deviation of 5.26%. Relative standard deviation (RSD) at each concentration level was calculated as standard deviation of response ratio DON peak area relative to CAF peak area /mean DON: CAF response ratio X 100. This was lower than previously reported (Trucksess et al., 1996 & 1998) due to inclusion of lower concentration standards.

DON recovery was determined from spiking blank wheat flour samples at 1.00 µg/g. Mean recovery for triplicate samples was 100.5% with range of 95.4 to 104.4%. Several



commercial flour samples were analyzed to acquire a blank sample that was used for sample development.

Figure 3.2. Representative HPLC chromatogram of wheat flour spiked with fungal culture material for DON concentration 0.25 µg/g and 30 µg CAF internal standard spike.

Culture material proved a consistent, inexpensive spiking source for internal standard curve development. Interfering components were adequately removed to detect low levels of DON and consistent CAF peak areas (Figure 3.2). Four sample runs were conducted with each concentration level in triplicate. The runs were conducted over two months and at least three days between runs. Statistical analysis revealed unequal variance across concentration levels increasing with greater DON concentrations in the samples. In order to account for this weighted least squares linear regression analysis was conducted. A weight was applied to the concentration (independent variable) that represented the inverse of squared standard deviation of the residual concentration. The internal standard curve could be described by the equation $y = 0.07854 + 0.68204x$ ($r = 0.983$). There was no significant difference between days so that the reduced model included only the concentration variable. Confidence limits (95%) were calculated from predicted values (Figure 3.3). Further method precision evaluation was conducted by utilizing the Horwitz ratio (Burgess, 2000) because runs had been performed over a wide time frame (Table 3.1). Our analysis followed published

findings that RSDs are greater in samples of lesser concentration (Whitaker et al., 2000). HORRAT values less than two are generally accepted as adequately precise (Burgess, 2000).

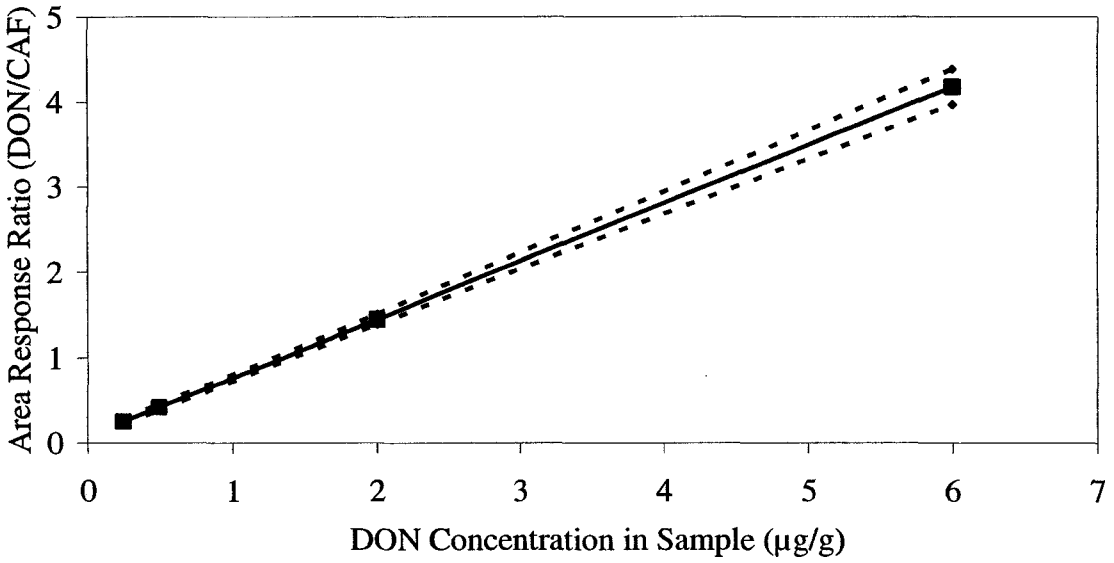


Figure 3.3. Response ratio (DON/CAF) predicted values and 95% confidence limits from weighted least square linear regression procedure. Calculations based on 12 samples per concentration level.

Table 3.1. Horwitz ratio (HORRAT) evaluation of method precision.

Sample Concentration µg/g	**Response Ratio DON /CAF area**	**Relative Standard Deviation Observed**	**Relative Standard Deviation Calculated**	**HORRAT**
0.26 ± 0.01	0.28 ± 0.07	25.7%	19.7%	1.31
0.51 ± 0.02	0.38 ± 0.07	18.7%	17.7%	1.06
1.99 ± 0.01	1.54 ± 0.18	11.5%	14.4%	0.80
5.96 ± 0.01	4.13 ± 0.53	12.7%	12.2%	1.04

Footnote: Mean and standard deviations of sample concentrations and response ratios of 12 samples per level. Relative standard deviation observed was calculated from standard deviation/ mean response ratio X 100. Relative standard deviation calculated was from RSD $= 2^{(1 - 0.5 \log C)}$, where C was relative concentration so that 100% has value of 1 or 10^0. HORRAT was the ratio RSD observed/RSD calculated. A HORRAT value of less than 2 was considered adequately precise.

This method was used to analyze commercial wheat flour and bread mixes acquired locally (Table 3.2).

Table 3.2. Commercial wheat-based sample analysis by HPLC following 30 µg CAF internal standard addition.

Sample Type	Sample Code	Response Ratio Area DON/Area CAF[a]	DON Concentration µg/g[b]
Wheat flour	GM	0.37	0.43
Wheat flour	WM	nd	nd
Bread mix	52B	2.11	2.98
Bread mix	52M	2.59	3.68
Bread mix	55M	0.24 ± 0.20	nd
Bread mix	36M	0.23 ± 0.10	0.28 to nd
Bread mix	99M	0.15 ± 0.21	0.26 to nd
Wheat	MW	3.80 ± 0.97	4.87 ± 0.93
Wheat[c]	PW	2.59 ± 0.70	14.45 ± 4.60

Footnotes: DON concentration calculated from linear regression of internal standard curve using fungal culture spiked wheat flour at 0.25, 0.50, 2.0 and 6.0 µg/g, 12 samples per concentration.

[a] Mean ± sd (where multiple sample analyses), nd indicated no DON peak detected to calculate response ratio.

[b] Mean ± sd (where multiple sample analyses), nd indicated concentration below limit of detection.

[c] Concentration calculation included adjustment for 5X sample dilution prior to analysis.

Due to an interest in low DON concentrations for subsequent research, a robust internal standard HPLC/UV method was developed reducing the limit of quantification. Caffeine proved suitable for an internal standard due to its absence from most food samples of interest and compatibility with current HPLC methods for DON sample preparation and analysis. This method reduced waste solvent and sample handling due to one analysis per

sample rather than two required with external standard addition. Wheat was the only matrix of interest currently therefore additional method validation would be required for other matrixes. This method utilized common laboratory reagents and proved robust over a range of DON contamination commonly observed in commercial wheat products.

CHAPTER 4. SENSITIVE SCREENING BIOASSAY FOR DEOXYNIVALENOL DETECTION IN FOOD SAMPLES

A paper to be submitted to *Food and Chemical Toxicology*

Cindy A. Landgren[1] and Suzanne Hendrich[2]

Abstract

A bioassay was used to screen for deoxynivalenol (DON) in commonly contaminated food samples. Six corn and six wheat samples acquired from local sources were tested. Samples were prepared by extraction of foods with aqueous acetonitrile and alumina: charcoal solid phase extraction. Human K-562 erythroleukemia cells, modeling immune stem cells, were incubated with sample extract and cell proliferation was assessed by MTS (dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) dye reduction assay. An aliquot of each sample was analyzed by HPLC for DON, 3-ADON (3-acetyldeoxynivalenol) and 15-ADON (15-acetyldeoxynivalenol), the latter two compounds not detected in any sample. DON was detected in one corn and two wheat samples by both methods with correlation of 0.97, $p < 0.0001$. Samples containing DON ranged in concentrations from 204 to 3792 ng DON/g food. Concentration resulting in 50% inhibition of proliferation (IC_{50}) for DON was 534 ng/ml media. Samples negative for DON by HPLC had bioassay responses as percent of controls ≥88%, with limit of detection of 140 ng/ ml (based on ±3 sd). This sensitive bioassay, as a model for immune cell progenitors, detected DON in a variety of food matrixes within the range of contamination present in the human food supply.

Introduction

Deoxynivalenol is a fungal secondary metabolite produced predominantly by *Fusarium graminearum* in a wide variety of cereal grains (Rotter et al., 1996). Other

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trichothecene mycotoxins such as 3-ADON and 15-ADON may be produced by the same fungal species and all are resistant to food processing (Hazel and Patel, 2004; Wolf-Hall et al., 1999). DON was the most common mycotoxin contaminant of food worldwide with estimated daily consumption of 0.78 to 2.4 μg/ kg bw per day from five GEMS/Food regional diets (Canady et al., 2001); the FDA has set an advisory level at 1 mg/kg in human food (Rotter et al., 1996). Many cell lines have been tested for cytotoxicity in response to purified trichothecenes (as reviewed by Gutleb et al., 2002; Jestoi et al. 2004). Human K-562 erythroleukemia cells were one of the most sensitive lines and have shown comparable sensitivity with human peripheral lymphocytes exposed to DON *in vitro,* median cytotoxic dose (CD_{50}) of 300 ng DON/ml media and median inhibition of phytohemagglutinin-induced blastogenesis of 430 ng DON/ml media (Visconti et al., 1991). The K-562 cell line originated from pleural effusion of a person with chronic myelogenous leukemia. K-562 cells are multipotent and can spontaneously differentiate into erythrocyte, granulocyte or monocyte progenitors although they have not been shown to express T or B lymphocyte markers (Lozzio et al., 1981). Minervini et al. (2004) proposed that the toxic mechanism of purified DON in K-562 cells was decreased cellular metabolism due to median proliferation inhibition dose (ID_{50}) of 651.2 ± 8.9 ng/ ml media by MTT [5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazol-2-yl)-3-(4-sulfophenyl) tetrazolium bioassay] and/or decreased cell proliferation with ID_{50} of 473.6 ± 148.0 ng/ml media by BrdU (5-bromo-2'-deoxyuridine) uptake assay rather than cell necrosis or plasma membrane damage, cytotoxic concentration > 24. 8 μg DON /ml media by Trypan Blue dye exclusion.

The objective of this study was to determine the sensitivity of human K-562 cells to extracts of commonly DON contaminated food samples for high throughput, economical, biologically relevant screening and correlate this bioassay with standard HPLC analysis.

Materials

DON (51481-10-8), 3-ADON (50722-38-8), 15-ADON (88337-96-6), HEPES buffer were purchased from Sigma Chemical (St. Louis MO); K-562 cells originally from ATCC; CellTiter 96® [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison WI). Dimethylsulfoxide (DMSO),

ethanol, acetonitrile (ACN), and methanol (MeOH) HPLC grade were from Fischer Scientific (Pittsburgh PA); Roswell Park Memorial Institute (RPMI) 1640, L-glutamine, gentamicin, heat inactivated fetal bovine serum (FBS) were from Gibco (Invitrogen Corp., Carlsbad CA).

Methods

Cell Culture

K-562 human erythroleukemia cells were cultured and maintained according to Visconti et al. (1991). Briefly cells were grown in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, HEPES buffer (10 mM) and gentamicin (50 μg/ml final conc.) (CRPMI). Cells were maintained at 5 X 10^5 cells/ml renewed with fresh media three times weekly in 75 cm^2 culture flasks, and incubated at 37°C with 5% CO_2 in air. Cells were prepared for assay by centrifuging for 7 min at 920g, counted with a hemocytometer with viability assessment by Trypan Blue dye exclusion (> 95% viability) prior to cell dilution in CRPMI to 2.5 X 10^5 cells/ml.

Sample Preparation

Standard solutions were made by diluting purified DON in CRPMI supplemented with 1.7% (v/v) ethanol and 0.3% (v/v) DMSO (Widestrand et al., 2003). This vehicle, without DON added, was the negative control media. Serial dilutions were made for final well concentrations of DON of 662.1 ng/ml to 82.8 ng/ml as confirmed by HPLC. Solutions were filtered through 0.22-micron cellulose acetate filters (Costar, Cambridge MA) prior to addition to microtiter plate wells.

Six corn (blue corn chips, flour and meals) and five wheat products (whole wheat noodles, baking mix, and flours) were purchased from local commercial sources. One additional whole berry wheat sample was a known naturally contaminated sample. All reusable glassware in contact with samples was oven baked for >3 hours at 180°C to destroy endotoxins. Food samples were ground if needed to pass a #20 screen and 25 g was extracted with 100 ml ACN: Milli-Q® water (84:16) blended at high speed for 3 minutes in ½ pint blender jars. The crude extract was filtered through a paper coffee filter and a six ml aliquot was applied to a 1.5 g alumina and ground charcoal (40:1) solid phase extraction column.

The eluate was collected and exactly 4.00 ml dried under a stream of N_2 in a 50°C water bath. The residue was dissolved in 800 µl DMSO/EtOH/CRPMI equating to 625 mg food sample per ml media.

HPLC Analysis

A second aliquot of sample crude extract was prepared as above except that the residue was dissolved in 800 µl 20% aqueous MeOH and filtered through 0.45-micron syringe filter (Alltech, Deerfield IL) into an autosampler vial. The HPLC system was a Hewlett Packard 1050 with UV photodiode array detector, autosampler, and degasser. Data acquisition and analysis was performed by ChemStation software. Analytes were separated using a YMC-Pack ODS-A C18 RP, S5µ 4.6 X 100 mm, with 4.6 mm guard column from Waters (Franklin MA). Samples, 50 µl injection, were analyzed by linear gradient 15 to 20% MeOH: DI water over 25 minutes with step up to 80% MeOH for 5 minutes then re-equilibration at 15% for 15 minutes, with flow rate of 0.7 ml/minute and total run time 50 min per sample. DON standard curve was determined in range 0.2 to 6.0 µg/g and ran each day of sample analysis. Analysis for 3-ADON and 15-ADON was performed similarly but mobile phase was 15% ACN: 85% DI water.

MTS Cell Proliferation Assay

Standard or sample media solutions (100 µl) were plated to 96 well flat bottom microtiter plates with serial 1:1 dilutions in sample media in triplicate, four dilutions per sample, followed by 100 µl of K-562 cell suspension (2.5×10^4 cells/ well). The plates were incubated for 46 hours at 37°C in 5% CO_2. Then 15 µl freshly thawed MTS reagent (CellTiter 96®) was added to each well. The plate was incubated for two hours and well absorbance read by a microplate reader (ELX808, Bio-tek Instruments, Inc.) at 490 nm.

Calculations and Statistical Analysis

The mean absorbance for each standard dilution or sample were blanked with the media control and converted to a percentage of the negative control [% of control response = (test mean response – mean blank)/ (negative control mean response – mean blank) X 100%]. The percentage of control response of DON standards was plotted against the DON concentration per ml of media to develop a standard curve. Food sample DON concentration was calculated from a linear regression equation of the standard curve. Analysis of variance

and student t-test were used to compare sample mean DON concentrations and negative controls. P values < 0.05 were considered significant. Three replicate experiments were conducted.

Results

The DON standard curve for the bioassay produced a median inhibition of cell proliferation (IC_{50}) at 534 ng DON/ml media, compared to control samples. One corn and two wheat samples were positive for DON in both the bioassay and HPLC analysis (Table 4.1). Positive samples in the bioassay had percent control response significantly different from the negative control ($p < 0.05$) at the highest concentration of food extract per ml media. Positive samples had peak area responses by HPLC analysis of greater than the limit of detection (LOD) of 93 ng/g food. The correlation between results of the two methods was 0.97, $p < 0.0001$ (Figure 4.1).

Table 4.1. Comparison of K-562 Bioassay and HPLC Detection of DON Concentration in Corn and Wheat Foods

Food Sample	Sample Origin	Bioassay[a] % control	Bioassay[b] ng DON/g food	HPLC[c] ng DON/g food
Cornmeal	IA	88.3 ± 3.9	nd[d]	nd[e]
Cornmeal	MN	55.4 ± 7.2	629.58 ± 349.0	386.67 ± 84.3
Cornmeal	IL	87.6 ± 1.0	nd	nd
Cornmeal	MO	88.1 ± 9.2	nd	nd
Blue corn chips	NB	95.4 ± 4.5	nd	nd
Corn flour	OR	93.8 ± 6.3	nd	nd
Wheat flour, white	IL	99.9 ± 3.9	nd	nd
Wheat flour, white	IA	104.9 ± 3.2	nd	nd
Wheat baking mix	CANADA	98.8 ± 6.7	nd	nd
Wheat flour, white	MN	80.9 ± 4.3	203.59 ± 33.0	288.96 ±17.8
Wheat, whole berries	MI	38.2 ± 1.0	3791.93 ± 282.4[f]	4364.68 ± 66.8
Wheat, whole grain noodles	MI	88.3 ± 7.1	nd	nd

[a] Mean percent of control K-562 cells (± SE), triplicate wells per experiment, three independent experiments, food extract equivalent to 625 mg/ml media.

[b] Calculated DON concentration (ng/g ± SE) in food based on purified DON standard curve (82.7 – 662.1 ng/ml), triplicate wells per plate.

[c] DON concentration (ng/g ± SE) by HPLC/UV on aliquot extract from each experiment (n=3).

[d] Not detected, within three standard deviations of control (LOD = 140 ng/ml).

[e] Below limit of detection of 93 ng/g based on 3:1 signal to noise ratio

[f] Calculation based on dilution 1:1 of extract for bioassay (n=2).

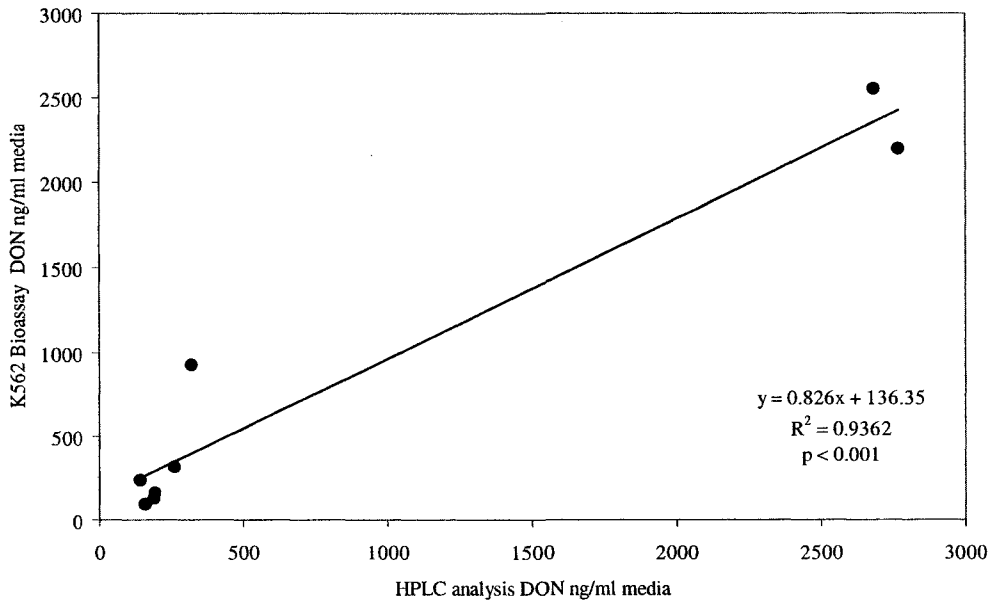

Figure 4.1. Correlation of K-562 bioassay and HPLC analysis for DON in food samples. Mean DON concentration of each positive sample, bioassay samples were mean percent of control for each plate (n =3) and HPLC analysis was one sample per experiment. The whole wheat berry sample had duplicate bioassay values because dilution was required to more accurately estimate DON concentration.

The wheat berry sample was highly contaminated and plated in the first experiment without dilution. Serial 1:1 dilution in the plate did not reduce the inhibition of K-562 cell proliferation by this sample thus the extract was diluted 1:1 prior to plate dilutions in the subsequent experiments to titrate toxin concentration. Corn not contaminated with DON was more inhibitory of cell proliferation than were uncontaminated wheat samples but there was no significant difference between the matrixes or from negative controls. At the highest extract concentration of 625 mg food sample equivalent/ml media, mean negative wheat samples showed effects on K-562 cell proliferation of 98.4 ± 2.4% of untreated controls and negative corn samples had mean cell proliferative response of 91.2 ± 2.0% of untreated controls (Figure 4.2). Other matrices tested in our lab had significant matrix effects on K-562

cell proliferation. Soy flour promoted cell proliferation causing 113.9 ± 6.3% of the cell proliferation found in the negative plate control whereas cell proliferation was inhibited by a rye and oat flour samples, 82.6 ± 4.1% and 36 ± 1.0% of negative plate controls respectively.

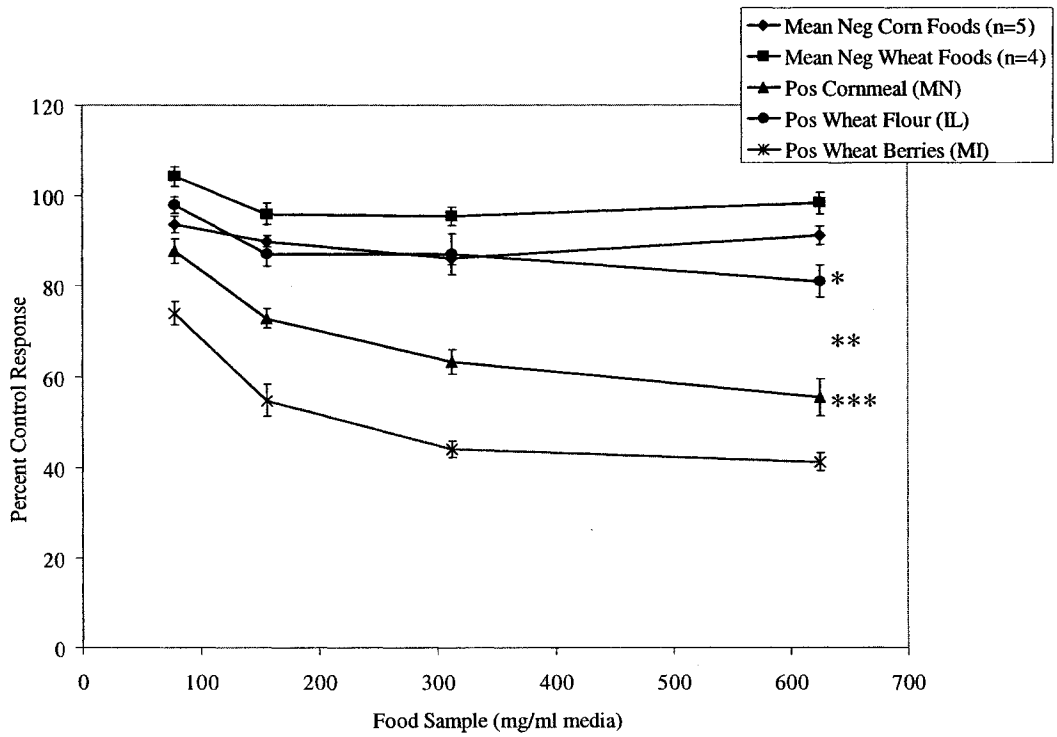

Figure 4.2. Inhibition of K-562 cell proliferation following 48-hr exposure to extracts of corn and wheat foods. Percent control response (± SE) was mean absorbance of triplicate wells per plate compared to untreated controls using the MTS dye reduction assay, three replicate experiments. Food sample amount was the dry weight of food per ml media represented in well dilutions.
* Significantly different (p= 0.02) from mean negative wheat samples by Student t-test.
** Significantly different ($p < 0.01$) from mean negative corn samples by Student t-test.
*** Significantly different ($p < 0.01$) from mean negative wheat samples by Student t-test.

Discussion

Numerous cell lines have been assessed for cytotoxicity due to DON exposure. The variability in sensitivity was dependent on cell line, detection assay, and matrix effects of sample screened. Recently work has focused on the 3T3 fibroblast cell line with bioassay development for screening cereal extracts comparing DNA synthesis with BrdU (Widestrand

et al., 2003). The BrdU was the most sensitive bioassay, IC_{50} 4.6 and 13 ng toxin/ml media, for these cells when compared to the MTT, IC_{50} 12 and 68 ng toxin/ml media, and LDH release, IC_{50} 18 and 42 ng toxin/ml media, assays based on response of 3T3 cells to purified T-2 and HT-2 toxin, respectively. 3T3 cells showed sensitivity to DON by the BrdU assay, IC_{50} 263 ± 18 ng toxin/ml media, but IC_{50} could not be determined for MTT or LDH release assays due to lack of sensitivity up to 2500 ng toxin/ml media (Widestrand et al., 1999). Although 3T3 cells are sensitive to some bioassay measurements, *in vivo* DON toxicity has not been identified in related tissue types such as cartilage or other connective tissue.

Visconti et al. (1991) compared DON cytotoxicity in K-562, MIN-GL1 Epstein - Barr virus transformed human B-cell line, and human peripheral lymphocytes using the MTT colorimetric bioassay. K-562 cells showed a CD_{50}, mean cytotoxic dose, of 300 ng purified DON/ml and ID_{50}, mean proliferation inhibitory dose, of 430 ng DON/ml in mitogen-stimulated human peripheral blood lymphocytes. Toxicity for 15-ADON was similar compared with DON in these cells although 3-ADON showed fivefold less toxicity than did DON. Immunotoxicity has been reported repeatedly for *in vitro* DON exposure to human peripheral blood cells (reviewed by Pestka and Smolinski, 2005) with particular sensitivity of human umbilical cord granulocyte progenitors, IC_{50} 8.9 ng DON/ml (Lautraite et al., 1997), therefore using a cell line of human immune system origin seemed relevant. Minervini et al. (2004) investigated cell viability by Trypan Blue dye exclusion, cell metabolism by mitochondrial MTT conversion, and cell proliferation by BrdU DNA incorporation in K-562 cells exposed to purified DON. Cell viability (>80% viability) was not affected by DON concentrations up to 84 μM (24.9 μg/ml media) whereas ID_{50} was 2.2 and 1.6 μM for the MTT and BrdU assays, respectfully. The mechanism of toxicity was proposed as inhibition of cell proliferation but not cell death. This mechanism seemed to hold for the K-562 cells in our bioassay since the highly contaminated wheat sample (3.7 μg/g) exhibited a maximum inhibition of cell proliferation of 35% of controls although microscopically cells appeared intact. As a practical bioassay, the K-562 cells had a range of sensitivity around the FDA advisory level of 1.0 μg DON/g food.

The MTT colorimetric bioassay has been used extensively to measure the mitochondrial dehydrogenase enzymatic reduction of tetrazolium salts into formazan that can

be detected spectophotometrically (Rotter et al., 1993). The absorbance value obtained is directly proportional to the cell population and mitochondrial metabolic activity. The MTS bioassay relies on similar enzymatic activity but the resulting formazan is water soluble, eliminating the requirement of the organic solvent crystal dissolution step in the MTT assay. When the MTT and MTS bioassays were compared for BHK-21 (baby hamster kidney) cell response to DON exposure, the cells were equally sensitive to both assays (Rotter et al., 1993). A further advantage of the MTS assay was continual color development in culture while MTT color development kills the cells, due to aspiration of media and addition of isopropanol, so underdevelopment cannot be resolved without repeating the bioassay. This comparison of bioassays was not conducted for our study but the one-step addition of MTS to test cell cultures would facilitate use of this bioassay for screening food samples in areas with limited access to technology, e.g., low-income countries. The bioassay also utilizes a lower level of resources than HPLC, only requiring refrigeration for media storage, incubation facilities, and standard sample preparation equipment.

Limited studies have screened food extracts for mycotoxins using bioassays and of these, fewer have investigated processed foods. Widestrand et al. (2003) applied cereal extracts from ground wheat, wheat bran, barley and oats to 3T3 cells and assessed cytotoxicity by BrdU DNA incorporation. They found some matrix effects in all extracts at maximum concentration of 400 mg grain equivalent/ml media but oats were cytotoxic in some samples at 100 mg grain equivalent/ml media. Other fungal metabolites may have been present but further work was needed to investigate the matrix effect of oats. Porcher et al. (1987) investigated matrix effects of corn extracts on murine splenocytes and LF hepatoma cells measuring [^{3}H] thymidine incorporation. These cells showed similar sensitivity to purified DON, IC_{50} 115 and 200 ng DON/ml media splenocytes and LF cells, respectively, but some naturally contaminated corn samples had inhibitory effects on both cell types that could not be explained by DON or other mycotoxins (T-2 toxin, HT-2, zearalenone, and diacetoxyscirpenol) analyzed by gas chromatography. We did not find significant inhibitory matrix effects in this series of experiments, using sample extracts equivalent to 625 mg grain/ml media, but in preliminary trials we observed cytotoxicity with other corn, oats, and rye food samples. Test samples were negative for 3-ADON and 15-ADON by HPLC/UV but

were not analyzed for other mycotoxins in our studies. Numerous other mycotoxins, including type A and B trichothecenes, zearalenone and related compounds, and/or fumonisin could have been present in these food samples. Extraction and clean-up of the food samples used in this study was specific for DON and its derivatives but similar sample preparation was conducted by Widestrand et al. (2003) where analysis for T-2 toxin, HT-2 toxin, nivalenol, and DON was conducted by gas chromatography with electron capture detection and 3T3 fibroblast MTT bioassay. They used the MycoSep™ #225 clean-up column which resulted in >80% recovery of tested mycotoxins. K-562 cells were sensitive to T-2 and HT-2 toxin with CD_{50} of 1 .0 and 20 ng purified toxin/ml media respectively (Visconti et al., 1991). K-562 cells were also tested for nivalenol sensitivity by this group and had similar CD_{50} of 300 ng/ml compared with DON and 15-ADON. Our study found strong correlation between bioassay detection and HPLC analysis with the same extract, therefore presence of immune-reactive amounts of other mycotoxins was not likely. Further analysis by GC or HPLC methods would verify absence of other mycotoxins.

Jestoi et al. (2004) investigated the cytotoxicity of organic and conventionally grown processed food extracts applied to feline fetal lung cells. DON, 3-ADON, and 14 others were analyzed in the samples by gas chromatography-mass spectroscopy and cytotoxicity was assessed by visual determination of cell death or inhibited growth. They found no correlation between the analytical mycotoxin concentration and cytotoxicity to the cells even though samples collected were not highly contaminated, maximum DON concentration 107 ng/g grain samples. They included foods that were highly processed such as ready-to-eat baby cereal and instant drink mix but no baked or extruded products. DON can be partially remediated by some food processing practices such as tortilla making or extensive milling (as reviewed by Hazel and Patel, 2004) but levels were stable or may be increased by other processes such as yeast bread production and brewing. Wolf-Hall et al. (1999) found DON to be stable in foods exposed to autoclave temperatures with significant enzymatic release of DON from the starch matrix by the addition of α-amylase to the sample before extraction, resulting in 3 to 30% greater DON recovery from corn grits and canned dog food, respectively. In some foods tested, DON may be underestimated if there has been extensive binding to the starch matrix of the contaminated cereals. The samples included in this trial

were not treated with α-amylase and only one sample had been heat treated, blue corn chips. Because the bioassay and HPLC DON concentrations were strongly correlated, binding of DON to carbohydrate molecules was not suspected. This aspect of DON binding in heat-treated foods should be considered in further development of this bioassay for other food samples.

The use of animals or other biological systems to evaluate the effects of a compound is the foundation of toxicology. Bioassays utilizing cell culture systems can evaluate biological effects in a controlled environment without the use of animals and mycotoxin screening fits well into this type of analysis (Buckle and Sanders, 1990). Screening for toxin contamination in a large number of samples with minimal analytical costs would be beneficial in regions epidemically contaminated with mycotoxins. This was illustrated by a report of broiler illness due to mycotoxin feed contamination correlated with cytotoxicity in a human epithelial cell line, HEp II, exposed to extracts of broiler feeds causing illness (Robb et al., 1982) with confirmation by TLC and GC. Strong correlation of bioassays with analytical methods further supports their utility. Bioassays could also provide evidence for loss of toxicity, such as testing decontamination strategies, prior to resource intensive animal studies. This bioassay utilizes an easy to maintain, nonadherent human cell line that exhibited proliferation inhibition upon exposure to purified DON and DON-contaminated extracts from commercially available foods. The range of detection was well below the FDA advisory level for human food and although there was an upper limit of detection (approximately 2.0 μg/g), DON concentrations in food samples could be titrated through dilution. This method had significant correlation with HPLC/UV, an established analytical method and could be performed in a similar timeframe for multiple samples. This bioassay could be performed as an inexpensive sensitive screening method where expensive analytical instrumentation was not available or correlation with *in vivo* activity was desired.

CHAPTER 5. LOW-LEVEL DIETARY DEOXYNIVALENOL AND ACUTE EXERCISE STRESS RESULT IN IMMUNOTOXICITY IN BALB/C MICE.

A paper to be submitted to the *Journal of Immunotoxicology*

Cindy A. Landgren[1], Marion L. Kohut[2], and Suzanne Hendrich[3]

Abstract

We hypothesized that acute exercise stress would exacerbate immunosuppressive effects of sub-acute exposure to dietary deoxynivalenol (DON). Male BALB/c mice were fed 0 or 2 mg DON/kg diet for 14 days, n=12 per dose, then exercised to fatigue on a treadmill. Mice were euthanized by decapitation, trunk blood and spleens were collected. Single cell suspensions of splenocytes were used to quantify immune function by plaque hemolysis and conconavalin-A (Con-A) stimulated lymphocyte proliferation assays. Serum corticosterone level was determined by enzyme immunoassay. Only the non-exercised DON-fed mice showed significant splenocyte proliferation suppression, 32.9 ± 17.9% of non-exercised controls ($p = 0.021$). Exercised controls and DON-fed exercised animals showed splenocyte proliferation of 68-75% of non-exercised controls. Antibody response to a T-dependent antigen, sheep red blood cells, was significantly less for exercised DON-fed mice than in controls ($p = 0.031$). Serum corticosterone levels were significantly higher for both exercised groups compared to the unexercised groups ($p < 0.001$). IL-4 secretion from mitogen-stimulated splenocytes was elevated by DON alone ($p < 0.05$) while IL-2 was elevated by DON with exercise stress ($p< 0.05$).

Our hypothesis was confirmed with respect to T-cell dependent antibody production, but not for splenocyte proliferation under Con-A stimulation. Exercise stress abrogated DON-mediated suppression of splenocyte proliferation, perhaps mediated by induction of elevated stress hormones counteracting cytokine expression alterations of DON.

Introduction

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Deoxynivalenol (DON) is the most common mycotoxin occurring in the food supply worldwide with most regional diets having less than 1 mg/kg (Pestka and Smolinski, 2005). High contamination can occur depending on environmental conditions permitting synthesis of the mycotoxin from *Fusarium* spp. commonly infecting wheat, corn, barley, oats, rye, and triticale (Rotter et al., 1996). Human intoxications were correlated with the high incidence of moldy crops (Bhat et al., 1989, Li et al., 2002) and DON was found in high concentrations in grains collected from these regions. Human illness (nausea, emesis, abdominal pain, bloody stools and increased incidence of secondary infections in children) has been suspected to be caused by DON due to its high incidence in foods in the affected area, low DON concentrations although high prevalence in areas where people were not affected, and low incidence and concentrations of other mycotoxins such as nivalenol, 3-acetylnivalenol and T-2 toxin. Epidemiologic studies have not been conducted but detection of DON in human urine as a biomarker of exposure (Meky et al., 2003) may prove useful to determine if human DON exposure is a disease risk factor.

Toxic effects of dietary DON have been studied in many species with swine being the most sensitive, emesis LOAEL 0.1 mg/kg bw, and others in the following order: mice = cats = dogs > rats > poultry > ruminants (Pestka and Smolinski, 2005). Toxic effects include emesis, reduced feed intake, reduced weight gain and immunotoxicity (Rotter et al., 1996). The immune system was sensitive to DON exposure since the replication required upon immune cell activation may be limited by the primary mechanism of action of DON, protein translational inhibition (Rotter et al., 1996). Mice are used extensively for evaluating xenobiotic effects on the immune system due to comparability with the human immune system and availability of reagents. Low-level dietary DON has had mixed effects on murine immune function depending on strain, dose and length of exposure. Tryphonas et al. (1986) found no significant effect on plaque-forming cell numbers (PFCs) or Con-A, lipopolysaccharide (LPS), or phytohemagglutinin P (PHA-P)-stimulated lymphocyte proliferation at 1, 2 or 4 mg DON/kg diet in Swiss-Webster mice fed for 5 weeks. Robbana-Barnat et al. (1988) reported significant reduction in serum levels of antibody to sheep red blood cells (sRBC) when weanling male BALB/c mice were fed 10 mg DON/kg diet for one or two weeks without significantly inhibited proliferation of splenocytes stimulated with

PHA or LPS. Forsell et al. (1986) found significant reduction of body weight after 24 days feeding of 2 mg DON/kg diet to B6C3F1 weanling female mice while white blood cell count was significantly reduced in mice fed 10 mg DON/ kg diet or greater. Greene et al. (1994) found reduced final body weight, rough hair coats and poor grooming in female and male B6C3F1 mice fed 2 mg DON/kg diet for 12 weeks. Transient significant reduction in erythropoetic activity in bone marrow and spleens occurred in Swiss-Webster mice fed 6.25 mg DON/kg naturally contaminated wheat-based diet for four weeks but parameters were not different from controls when mice were fed up to 18 weeks (Arnold et al., 1986). Mice were sensitive to DON for various parameters but 2 mg/kg in the diet appears to be the overall LOAEL for sub-chronic studies.

Limited work has investigated interactions of DON with other xenobiotics or stresses on the immune system. Islam et al. (2002) found significant potentiation of glucocorticoid release and trichothecene-induced lymphocyte apoptosis in mice due to a single LPS (0.1 mg/kg bw i.p.) and simultaneous DON exposure (12.5 mg/kg bw oral).

Running to exhaustion on a treadmill by mice was an established model to study acute exercise stress. Hoffman-Goetz et al. (1988) determined that this model of stress produced significant elevation of serum corticosterone in C57BL/6J mice but no significant change in lymphocyte proliferation with LPS or pokeweed mitogen stimulation compared to sedentary controls. Acute exercise stress in C3He mice caused a significant reduction in the percent splenic Ig+ B-cells but not in T-helper, T-suppressor or total T-cells (Hoffman-Goetz et al., 1989) however when splenocytes were incubated with Con-A, T-suppressor cells increased significantly and the B cell effect was not observed (Randall Simpson et al., 1989). Pedersen et al. (1997) proposed more significant immunosuppressive contribution from elevated catecholamine and growth hormone in immediate response to exhaustive exercise and a lag of immunomodulatory effects of glucocorticoids. To our knowledge there are no reported investigations of acute exercise fatigue interaction with any xenobiotic immunotoxicant.

The purpose of this study was to investigate interactive effects of two commonly encountered modulators of the immune system using a murine model. We investigated a

dietary concentration of DON which could occur in the human food supply and measured endpoints of immunotoxicity and an indicator of acute exercise fatigue.

Materials

DON was from Sigma (St. Louis MO). Diet components were from Harlan Teklad (Madison WI) and ICN Biomedical Inc. (Irvine CA). HBSS (Hank's balanced salt solution) and AIM-V® (Adoptive Immunotherapy Media) media was from GIBCO (Invitrogen Corp., Carlsbad CA). Guinea Pig Complement Low-Tox® was from Accurate Chemical Scientific Corp. (Westbury NY). OCTEIA® Corticosterone (enzymeimmunoassay) was from ALPCO Diagnostics (Windham NH). Sheep red blood cells were from National Veterinary Service Laboratory (Ames IA).

Methods

Animals

The animal protocol was reviewed and approved by Iowa State University Committee on Animal Care. Twenty-four male 8-week-old BALB/c mice were purchased from Harlan (Indianapolis IN). They were housed individually in shoebox cages with hardwood shavings and acclimated for 7 days on AIN-93G diet (Reeves et al., 1993). They were fed ad libitum, water changed three times a week and exposed to reversed12 hour light/dark cycle at 72°F and 20% humidity. All animals were trained twice on the treadmill during the acclimation period for 15 min at 10 m/min. Following acclimation all animals were weighed and blocked into three groups by weight then randomly assigned within each block to the treatment groups, 2 mice per treatment within each block. The treatment groups were: No DON/ No exercise, 2 ppm DON/ No exercise, No DON/ Exercise, and 2 ppm DON/ Exercise. DON was added to the diet at 2 mg/kg from standard solution (500 μg/ml in water). DON concentration in the diet was confirmed by HPLC analysis in our laboratory. The diet was formed into biscuits by mixing the dry diet with adequate water to form dough, which was rolled out, scored and allowed to dry on screen racks at room temperature until firm, about two days. Food was stored in sealed plastic bags at 4°C until use. Food consumption was measured daily for all animals. DON standards and contaminated equipment was handled

with use of personal protective equipment. Decontamination of glassware and diet mixing equipment was by soaking in 10% hypochlorite prior to cleaning.

Exercise Stress

The animals were exercised by block, one block per day on three consecutive days and order was determined randomly. The non-exercised mice in the block were exposed to the noise of the treadmill by placing them in close proximity. The exercise started in the morning toward the end of the set dark cycle to occur during their active nocturnal period. Animals were placed in individual lanes and the treadmill speed was started at 10 m/min with speed increased by 2-3 m increments every 20 min, maximum speed attained was 20 m/min. Mice were removed from the treadmill when they could no longer maintain pace with physical prodding and returned to their cages for 30 min with access to food and water. Time to fatigue was 2.5 to 4.25 hours.

Tissue Collection

Individual cages were transported to the necropsy room where animals were decapitated within 2 min of picking up the cage. Trunk blood was collected, spleens removed aseptically and immediately placed in stomacher bags containing 10 ml HBSS and processed until tissue dissolution by stomacher laboratory blender (Tekmar, Cincinnati OH). Single cell suspension was achieved by filtering through sterilized nylon mesh and washing cells once in HBSS before suspending in AIM-V® media. Cell viability was determined by Trypan Blue dye exclusion (>95% for all animals) and concurrent cell counting by hemocytometer. Blood was allowed to clot at room temperature, centrifuged at 2500 rpm for 10 min, and serum separated and frozen at -20°C as soon as possible.

Lymphocyte Proliferation

Spleen cell concentration was adjusted to 5 X 10^6 cells/ml with AIM-V® media and 100 µl was pipetted into six replicate wells per animal in 96 well round bottom plate, Con-A added to three wells (1 µg/well, 10 µl) and three wells remained as controls. The plate was incubated at 37°C, 5% CO_2 for 72 hours. CellTiter 96® (Promega, Madison WI) 10 µl was added to all wells and incubated for 60 minutes at 37°C, when well absorbance was read at 450 nm in microplate reader (Benchmark™, Biorad Corp., Hercules CA). Mean absorbance of Con-A treated wells and control wells for each subject was calculated, difference

determined and compared to the control treatment group to assess percent inhibition of lymphocyte proliferation.

Hemolytic Plaque Assay

This assay quantifies the number of B-cells activated to produce IgM in response to a T-cell dependent antigen, sRBC. All mice were primed four days prior to sacrifice with 0.20 ml intraperitoneal injection of 20% sRBC, vol/vol in PBS, which had been washed three times with PBS. Spleen cells were diluted with AIM-V® media to 3 X 10^6 cells/ml. Sheep RBC (4%) suspension in AIM-V® media was prepared the day of assay by washing sRBC three times with PBS prior to dilution to final concentration. Guinea pig complement was absorbed to sRBC by rehydrating the lyophilized pellet with 1 ml sterile water then mixing with 250 µl washed packed sRBC in a microcentrifuge tube, incubating on ice for 10 min, centrifuging at 1500 rpm for 10 min and decanting the complement, repeating 3 times. Aliquots of the absorbed complement were stored at -20°C until day of use when it was thawed and diluted 3:1 with AIM-V® media within 20 min of use. The plaque assay was conducted as per Cunningham and Szenberg (1968). Double-sided microscope slide chambers were constructed the day before the assay. The following components were mixed in 1.5 ml microcentrifuge tubes: 50 µl spleen cell suspension, 50 µl 4% sRBC, and 50 µl absorbed diluted guinea pig complement. Duplicates of each subject were performed and one sample using AIM-V® media instead of complement was the negative control. From each replicate 50 µl was pipetted into each side of one slide assembly, three slide assemblies per subject, 4 test chambers and 2 controls. Slide edges were sealed with melted paraffin and incubated in humidified boxes for 60 min at 37°C. Lysing of sRBCs in response to the interaction of complement and IgM produced plaques, with a single plaque-forming cell (PFC) in the center, that were counted in each chamber under 4X magnification. Mean PFCs were calculated from 4 chambers per subject. Mean plaques times 20 equaled PFCs per 10^6 spleen cells.

Serum Corticosterone

This assay was conducted according to the manufacturer's instructions for the OCTEIA kit. Briefly 30 µl of thawed mouse serum was diluted with PBS containing horse serum 1:10. Included calibrators, controls and diluted mouse samples were pipetted (100 µl)

in duplicate into provided antibody coated microplate. Enzyme conjugate, corticosterone labeled with horseradish peroxidase, was added to each well (100 µl) and the plate was incubated for 18 hours at 4°C. The plate was washed three times manually with PBS supplemented with 0.05% Tween and tapped dry. Tetramethylbenzidine (TMB) was added to each well (200 µl) by multichannel pipettor. The plate was incubated for 30 min at room temperature then 100 µl of hydrochloric acid stop solution was added to all wells. Well absorbance was measured at 450 nm by a Biorad microplate reader. A standard curve was constructed with calibrators and serum concentration of corticosterone was read from the curve and corrected for 10X dilution factor.

Cytokine Analysis

Spleen cells, 5 X 10^6 cells/ml in AIM-V media, were pipetted into 24-well flat bottom plates, 1 ml per well. Three wells per subject were stimulated with Con-A (1 µg/ml) and two were unstimulated control wells. Supernatants were collected from wells at 24 and 48 hours for IL-2, IL-4, and IFN-γ determination. Supernatant aliquots were frozen at -20°C until batch ELISA was conducted. Cytokine concentration was determined using mouse monoclonal antibodies in kits (PharMingen, San Diego CA) and following manufacturer's instructions. Briefly, 96-well ELISA plates (Costar, Corning NY) were coated with capture antibody for IL-2, IL-4 or IFN-γ overnight at 4°C. Plates were blocked with PBS-10% heat-inactivated fetal bovine serum for one hour at room temperature before the addition of standards and thawed supernatants (100 µl) and incubation at room temperature for two hours. Plates were washed in plate washer between each step with PBS-0.05% Tween. Biotinylated anti-mouse cytokine monoclonal antibody with avidin-horseradish peroxidase conjugate was added to each well (100 µl) and plates were incubated for one hour at room temperature. Plates were washed prior to addition of TMB and hydrogen peroxide substrate, incubation in the dark for 30 min, and read at 655 nm on a Biorad microplate reader. Cytokine concentrations were determined from best fit linear regression of blanked mean absorbance of standards against standard dilutions.

Statistics

One-way analysis of variance was conducted with SAS with student T-test for pair-wise contrasts. Cytokine comparisons were evaluated by repeated measures analysis with

Tukey-Kramer adjustment for post-hoc pair-wise comparison in SAS (SAS 9.1, Cary NC). P value <0.05 was considered significant.

Results

There were no significant differences between treatment groups for initial (22.9 ± 1.1 g) or final body weight (24.5 ± 1.0 g), average daily feed intake (3.25 ± 0.22 g), body weight gain (1.6 ± 0.8 g), or total spleen cells (2.21 ± 0.70 X 10^8).

A significant difference was found between the treatment groups for Con-A stimulated lymphocyte proliferation (Figure 5.1). A possible error occurred in Con-A dilution for one block of animals resulting in cell death and plate exclusion, therefore n=4 for each treatment group. Mice fed 2 mg DON/kg and not exercised had significant inhibition of proliferation when compared to the control group.

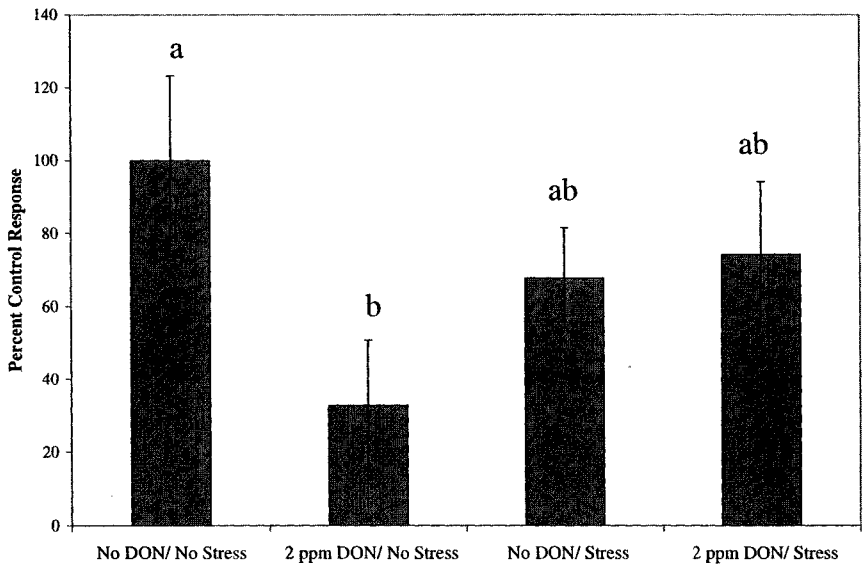

Figure 5.1. Inhibition of Con-A stimulated splenocyte proliferation in response to sub-acute dietary DON. Spleen cells (5 x 10^6/ml), from BALB/c mice after completion of acute exercise fatigue +/- 2 mg/kg dietary DON for 14 days, were incubated with Con-A for 72 hours. Proliferation was assessed by MTS dye reduction assay. Values are mean ± SEM. Different letters significantly different from each other, p = 0.021, n = 4 per treatment.

There was significant reduction of B-cells expressing antibodies specific for sRBC in mice fed 2 mg DON/ kg diet and exercised compared to the control group and a trend toward

a significant main effect of exercise stress (p = 0.06) (Figure 5.2). Manual enumeration of spleen cells was not consistent across all blocks because the counts were done by two different technicians, thus one block was excluded from these results.

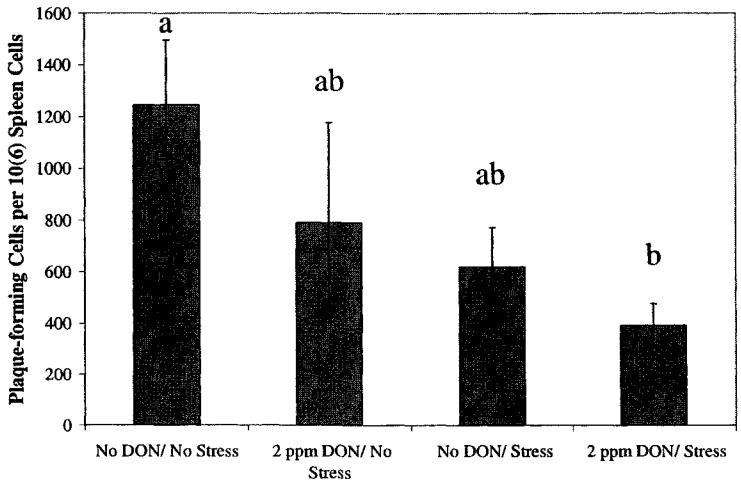

Figure 5.2. Interaction of 2 mg/kg dietary DON and acute exercise stress inhibits plaque-forming cells in spleen to sRBC. Spleen cells (3 X 10^6 cell/ml) from BALB/c mice after completion of acute exercise fatigue +/- 2 mg/kg dietary DON for 14 days were assessed for number of PFCs by Jerne-Salzberg plaque assay. Mean PFCs ± SEM based on four chambers per mouse. Different letters significantly different from each other, p = 0.031, n = 4 per treatment.

There was a significant main effect of stress on serum corticosterone levels (Figure 5.3). ELISA analysis of supernatant from Con-A stimulated splenocytes revealed a significant increase in IL-4 secretion after 48 hours of culture in cells harvested from DON fed non-exercised mice compared to all other treatments (Figure 5.4). IL-2 was significantly elevated from DON-fed stressed mice compared to either non-stressed groups after 48 hours culture (Figure 5.4). No treatment differences in IL-2 or IL-4 expression were observed after 24 hours culture or for IFN-γ at either time point.

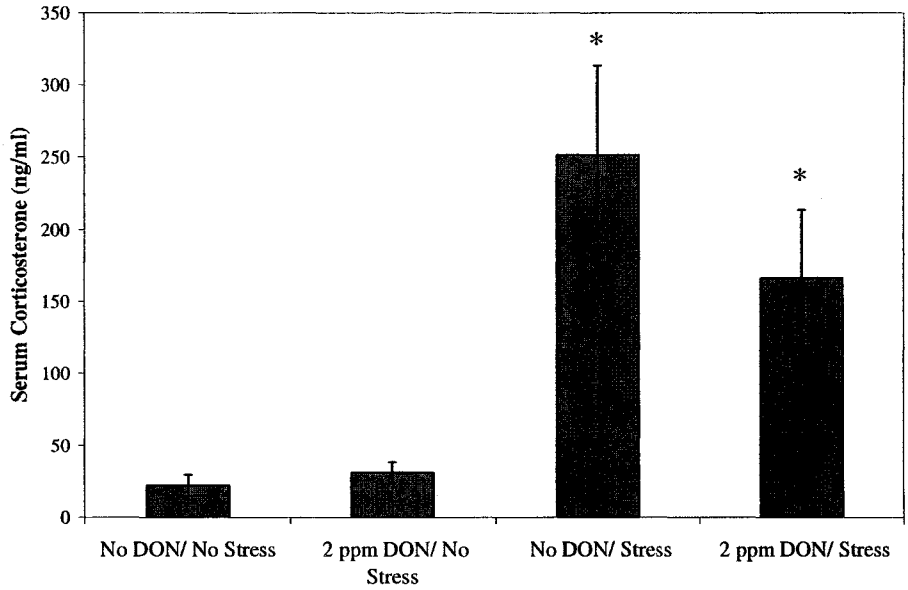

Figure 5.3. Serum corticosterone significantly elevated by acute exercise stress in BALB/c mice. Serum corticosterone determined by enzyme immunoassay from blood collected 30 minutes after completion of acute exercise fatigue of BALB/c mice +/- 2 mg/kg dietary DON for 14 days. Values are means ± SEM, n = 6. *Significant main effect of acute exercise stress ($p < 0.001$).

Discussion

BALB/c mice were chosen for our study because they had shown sensitivity to DON at low dietary doses and are used commonly in immunotoxicity studies. BALB/c male mice have been used in two DON feeding studies (Robbana-Barnat et al. 1987 & 1988) where the earlier trial found significantly reduced food intake with 2.5 mg/kg dietary DON ($p < 0.05$, n = 12) for one week with the second study finding a trend toward reduced weight gain and reduced anti-sRBC serum antibodies but no effect on feed intake in the 5 mg DON/kg diet group (n = 8) for one or two week feedings. There were no apparent differences between these two studies by the same laboratory indicating sensitivity of this mouse strain but potential variability within the strain. The dietary dose chosen for our study seemed likely to produce an observable effect without overt clinical illness.

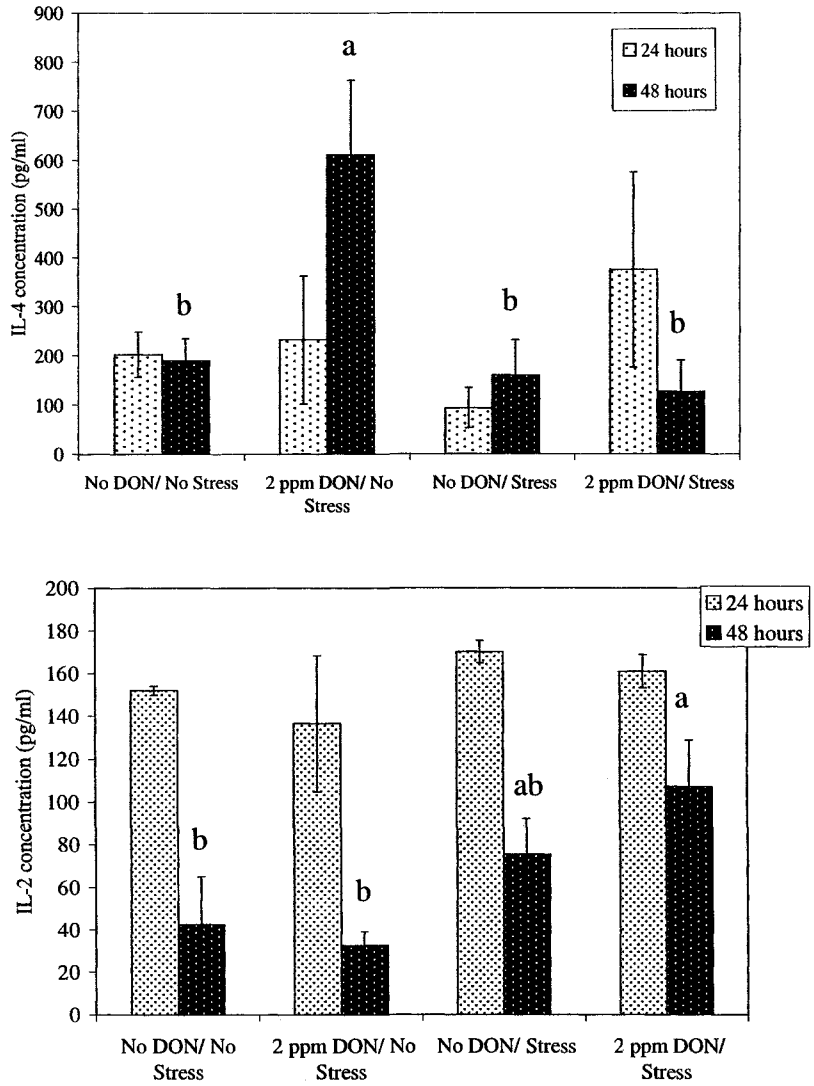

Figure 5.4. Dietary DON inhibits IL-4 and stimulates IL-2, with acute exercise stress, in Con-A stimulated spleen cells. Con-A stimulated spleen cell supernatants, from BALB/c mice after completion of acute exercise fatigue +/- 2 mg/kg dietary DON for 14 days. IL-4 and IL-2 determined by murine monoclonal antibody ELISA. Values are mean ± SEM for 2 replicates per subject, n = 4 mice per treatment. Different letters significantly different from each other at 48 hours incubation ($p < 0.05$). No significant different between treatment groups at 24 hours incubation.

Significant inhibition of proliferation of spleen cells stimulated by Con-A, a polyclonal T-cell mitogen, in mice fed DON and not exercised might be due to the effect of elevated IL-4 since this cytokine promotes differentiation of B-cells and inhibits Th1 cells (Elenkov and Chrousos, 2002). IL-4 has been shown to be expressed *in vitro* from Con-A

stimulated CD4+ T-cells from female B6C3F1 mice after 7 days incubation with 100 ng DON/ml media (Ouyang, et al., 1995). Rat peripheral blood lymphocyte proliferation following PHA mitogen-stimulation was inhibited by 50 and 100 ng DON/ ml media (Miller and Atkinson, 1986). Protein synthesis, assessed by [^{14}C]leucine incorporation assay, and proliferation, measured by [^{3}H]thymidine incorporation, of LPS-stimulated splenocytes from female B6C3F1 mice was inhibited by 100 ng DON/ml media (Warner et al., 1994) with elevated IL-4 in macrophage depleted lymphocytes with 25 to 100 ng DON/ml. This study indicated that protein synthesis inhibition occurred concurrent with increased secretion of IL-4 in response to DON exposure *in vitro,* although the *in vivo* situation was much more complicated, this finding may help to explain the results observed in our study.

Acute exercise ameliorated the effect of DON on splenocyte proliferation, perhaps because exercise was known to affect T cell subpopulations. Mice exhaustively exercised had significantly increased percentage of T-suppressor splenocytes when stimulated in culture with Con-A (Randall Simpson et al., 1989). IL-2 was significantly elevated in our study by dietary DON + exercise stress compared to non-exercised mice. This seemed reasonable since lymphocyte proliferation was not different from control and IL-2 is a potent T-cell and NK cell growth factor (Handa, et al., 1983). Increased percentage T-suppressor cells in response to catecholamine induced lymphocyte trafficking (Pedersen and Hoffman-Goetz, 2000) could be responsible for this IL-2 release. DON has stimulated IL-2 release and increased expression of IL-2 mRNA after 10 ppm dietary DON for 4 weeks in male B6C3F1 mice (Zhou et al., 1998), lower doses were not tested. Increased IL-2 may be an additive effect of DON and acute exercise stress.

Our results indicated significant inhibition of PFCs by 2 mg DON/kg diet with acute exercise stress, which has not been reported at this dose in mice or any other species. Plaque forming cells are differentiated B-cells producing mostly IgM since primary immunization of antigen was 4 days prior. Sheep RBCs are T-cell dependent antigens thus for antibody expression the antigen must be processed by antigen presenting cells, MHC II recognition and activation of T helper cells, and antigen presentation for B-cell activation (Abbas et al., 1996). Numerous cytokines must also be produced by these cells to promote activation, differentiation, and clonal expansion of the different cell populations. IL-2 has an inhibitory

feedback on Th2 cells which may have been additively stimulated by DON and acute exercise stress. DON and other trichothecene mycotoxins inhibit protein synthesis (Rotter et al., 1996) which may further inhibit the ability of cells to produce antibody although cytokine production may stimulated. The same acute stress model in male C3He mice resulted in a trend toward decreased Ig+ cells in splenic cultures with Con-A (Randall Simpson et al., 1989). Conversely stress hormones such as glucocorticoids and catecholamines can inhibit release of cytokines (IL-12, IL-1) from antigen presenting cells inhibiting cellular and promoting humoral immune functions (Elenkov and Chrousos, 2002) as well as inhibit end stage differentiation of B-cells (Madden et al., 1995). Elevated plasma corticosterone confirmed significant stress in the acutely exercised mice in our study. New cytokines have been discovered recently (IL-23, IL-27) that have overlapping functions with IL-12 (Agnello, et al., 2003), but their response to stress hormones has not yet been revealed.

The interactive effect observed in our study could be a combined effect of acute exercise-induced reduction of B-cell numbers and/or inhibited end-stage differentiation in the spleen, altered cytokine expression and/or secretion, inhibitory or stimulatory. Phenotypic evaluation of spleen cell populations with a cytokine assessment battery would define this interaction more clearly. To date, inhibition of synthesis of specific proteins by DON has not been determined. Genomic or proteomic global investigation to identify possible high affinity protein targets is warranted.

Conclusions

To our knowledge, this is the first report of an immunotoxic interaction between a low-level dietary exposure to DON, or any other xenobiotic, and acute exercise stress. Acute exercise stress may protect the mouse from DON immunotoxicity due to differential effects on lymphocyte subsets and cytokine cell signaling proteins. Acute exercise stress interacted with dietary DON to cause a significant reduction in PFCs, which may be a result of stress hormone activity and DON effects on one or more cells involved in the T-dependent response to sRBCs. Effects seen in this study were observed at a lower level of DON than has been previously reported and may reflect special sensitivity in BALB/c male mice.

Further investigation on immune function is warranted with this model of two commonly occurring human stressors, acute exercise and low level dietary DON.

CHAPTER 6. IMMUNOMODULATORY EFFECTS OF LOW-DOSE DIETARY DEOXYNIVALENOL AND ACUTE EXERCISE STRESS IN BALB/C MICE

A paper to be submitted to *Toxicological Sciences*

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Abstract

Dietary deoxynivalenol (DON) dose response and interaction with acute exercise stress was investigated in male BALB/c mice with emphasis on alteration of functional immune parameters. Mice were fed 0, 1 or 2 mg DON/ kg diet for 28 days then half of each dose group were exercised to fatigue on a treadmill. DON at 2 mg/kg diet inhibited weight gain ($p < 0.0002$), red blood cell counts, and hematocrit but increased feed intake ($p = 0.0003$). Blood lymphocyte count was suppressed by DON feeding ($24.9 \pm 4.8\%$) but more potently reduced by acute stress ($57.5 \pm 2.1\%$, $p < 0.05$). DON at 1 mg/ kg diet significantly stimulated splenocyte NK cytotoxicity (156%, $p < 0.01$), PFCs (121% control, 155% of 2 ppm, $p < 0.05$), and spontaneous interferon-γ secretion ($p < 0.05$). Splenocyte IL-4 was stimulated in mice stressed alone compared to control or 1 mg DON/kg diet alone. Significant inhibitory effects of acute exercise were observed for spleen to body weight ratio ($p < 0.0001$), hematocrit ($p = 0.017$), hemoglobin ($p < 0.0001$) and NK cell cytotoxicity ($p < 0.05$). Exercise stress increased blood neutrophils (173.6%, $p < 0.0003$) and neutrophil to lymphocyte ratio (348%, $p < 0.0001$). This is the first report of *in vivo* immunostimulatory effects of sub-chronic low dose DON feeding promoting both cellular and humoral responses through differential cytokine production, which may be abrogated by acute exercise stress. Apparent immunological hormesis of feeding 1 mg DON/kg diet deserves further investigation.

Introduction

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Deoxynivalenol (DON) contamination of food crops (wheat, corn, barley, oats, rye) occurs worldwide and humans can be commonly exposed to levels ≤ 1 mg/kg in foods but can encounter contamination as high as 5 to 20 mg/kg (Pestka and Smolinski, 2005). Human intoxications have been reported that were correlated with the high incidence of moldy crops (Bhat et al., 1989; Li et al., 2002). Occurrence of human illness, exhibited as gastrointestinal distress in adults and increased upper respiratory tract infections in children, was suspected to be caused by the prevalence of DON contamination of foods in the affected area with concomitant low incidence and concentrations of other mycotoxins such as nivalenol and T-2 toxin. Epidemiologic studies have not been conducted but presence of DON in human urine was proposed as a biomarker for DON exposure (Meky et al., 2003) and may prove useful to determine if DON exposure is a risk factor for human clinical illness.

Toxic effects of dietary DON have been studied in many species with swine being the most sensitive, emesis LOAEL 0.1 mg/kg bw, reduced weight gain LOAEL 0.07 mg/kg bw, but dogs, emesis LOAEL 0.3 mg/kg bw, and mice, reduced weight gain LOAEL 0.3 mg/kg bw, inhibition lymphocyte proliferation LOAEL 0.5 mg/kg bw, appear similarly sensitive with others in the following order: rats > poultry > ruminants (Pestka and Smolinski, 2005). DON is a protein translation inhibitor and the immune systems' high mitotic requirement may contribute to sensitivity to this toxin (Rotter et al., 1996).

The murine model was used extensively for evaluating xenobiotics' effect on the immune system due to similarity of immune responses with the humans and availability of reagents. Dietary exposures of less than 10 mg DON/kg diet have had mixed effects on murine immune function depending on strain, dose, length of exposure and method of assessment. Tryphonas et al. (1986) found no significant effect on antigen specific plaque forming cells (PFCs) or Concanavalin-A (Con-A), lipopolysaccharide (LPS), or phytohemagglutinin P (PHA-P) mitogen-stimulated splenocyte proliferation in Swiss-Webster male mice fed 1, 2 or 4 mg DON/ kg diet for 5 weeks. The same strain fed 6.25 mg DON/ kg diet for 4 weeks had reduced bone marrow and splenic red blood cell production (Arnold et al., 1986). Weanling male BALB/c mice fed 10 mg DON/ kg diet for one or two weeks had significantly reduced serum antibody titers to sheep red blood cells (sRBC) and inhibited PHA and LPS stimulated splenocyte proliferation, no effect was observed in mice

fed 5 mg DON/ kg diet (Robbana-Barnat et al., 1988). Dietary DON at 2 mg/ kg diet for 24 days resulted in significant body weight reduction in B6C3F1 weanling female mice while 10 mg/ kg resulted in significantly decreased white blood cell counts (WBC) (Forsell et al., 1986). Female and male B6C3F1 mice fed 2 mg DON/ kg diet for 12 weeks had reduced final body weight, rough hair coats and poor grooming (Greene et al., 1994). BALB/c male mice exposed to 2 mg DON/ L in drinking water for 2 weeks exhibited reduced splenic clearance of *Salmonella enteritidis* 7 days after oral exposure (Hara-Kudo et al., 1996) indicating impaired host resistance at low level DON exposure.

Limited work investigated interactions of DON with other xenobiotics or stressors on the immune system. Islam et al. (2002) found significant synergistic glucocorticoid-dependent lymphocyte apoptosis following acute concurrent LPS (0.1 mg/kg bw ip) and DON (12.5 mg/kg bw) exposure in male B6C3F1 mice.

Exhaustion of mice by treadmill running was an established animal model to study the effects of acute exercise stress. Hoffman-Goetz et al. (1988) measured significant elevations of serum corticosterone without significant change in lymphocyte proliferation with LPS or pokeweed mitogen stimulation compared to sedentary controls in C57BL/6J mice using this model. Acute exercise stress in C3He mice caused a significant reduction in the percent of Ig+ B-cells but no effect on T lymphocyte subsets in the spleen (Hoffman-Goetz et al., 1989) but when splenocytes were incubated with Con-A, T-suppressor cells increased significantly and the B cell effect was not observed (Randall Simpson et al., 1989). Azenabor and Hoffman-Goetz (1999) reported imbalance of oxyradical production and antioxidant enzyme defenses in the thymus and spleen after a bout of exhaustive exercise in C57BL/6 female mice. Pedersen et al. (1997) proposed more significant immunosuppressive contribution from elevated catecholamine and growth hormone in immediate response to exhaustive exercise and a lag of immunomodulatory effects of glucocorticoids. We found inhibition of PFC response to 2 mg DON/ kg diet and acute exercise stress in BALB/c mice [Landgren et al., (2005) Low-level Dietary Deoxynivalenol and Acute Exercise Stress Result in Immunotoxicity in BALB/c Mice, manuscript in progress]. To our knowledge there are no other reports of acute exercise fatigue interaction with a xenobiotic immunotoxicant.

The purpose of this study was to investigate interactive effects of two commonly encountered immunomodulators conducting a battery of Tier I immune function tests as proposed by Luster et al. (1988). We hypothesized an interactive dose-responsive immunosuppression between low-dose dietary DON and acute exercise stress that would be characterized as impaired humoral immune function and cytokine alterations.

Methods

Materials

Pretrial rodent chow, cornstarch, casein, dextran cornstarch, sucrose, soybean oil, fiber, AIN-93G mineral and vitamin mixes were from Harlan Teklad (Madison WI). Choline bitartate and L-cysteine were from ICN Biomedical Inc. (Irvine CA). Recombinant human IL-2 (rhIL-2), HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid], and EDTA (ethylenediaminetetraacetic acid) were from Sigma, St. Louis MO. Hank's balanced salt solution (HBSS), Roswell Park Memorial Institute (RPMI 1640) and Adoptive Immunotherapy Media (AIM-V®) media were from GIBCO (Invitrogen Corp., Carlsbad CA). Guinea Pig Complement Low-Tox® was from Accurate Chemical Scientific Corp. (Westbury NY). Sheep RBCs were from Remel Microbiology Products, Lenexa KS. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] CellTiter 96® was from Promega Corporation, Madison WI.

Animals

The animal protocol was reviewed and approved by Iowa State University Committee on Animal Care. Fifty-four 6 – 8 week old male BALB/c mice were purchased from Harlan (Indianapolis IN). They were housed individually in shoebox cages with hardwood shavings, fed rodent diet *ad libitum*, water changed three times a week and exposed to 12-hour reversed light/dark cycle at 72° F and 20% humidity. Mice were fed semi-purified AIN-93G diet beginning at 11-13 weeks of age. An acclimation period of 7 days on control diet occurred prior to 28 d DON feeding. Animals were weighed weekly and observed daily for any signs of illness. All animals were trained twice on the treadmill during the acclimation period for 15 min at 10 m/min. Animals were divided into three blocks by weight and randomly assigned within each block to the treatment groups, 3 mice per treatment within each block. Animals were fed 0, 1 or 2 mg DON/ kg diet, n= 18/ dose; half the animals at each dose were

exercised to exhaustion. DON was added to the diet from autoclaved culture material which was prepared by solid culture of *Fusarium graminearum* [R-5245 (USA, OH), Fusarium Research Center, PSU, University Park PA 16802] per Witt et al. (1985). Briefly 350 g commercial converted rice was autoclaved with 150 ml deionized water then inoculated with 10^6 macroconidia, from CMC media starter liquid culture, and incubated for 20 days at 28°C. Culture was autoclaved, air dried and ground to a meal for storage at -20°C. Diets were analyzed by HPLC for confirmation of DON levels. Culture material was analyzed (NDSU, Fargo ND) and found to contain 123 mg/kg DON, 112 mg/kg 3-acetyldeoxynivalenol (3-ADON) but no other mycotoxins. AIN-93G semi-purified diet (Reeves et al., 1993) with and without DON was formulated into biscuits by mixing the dry diet with adequate water to form dough, which was rolled out, scored and allowed to air dry at room temperature until hard (less than 2 days). Feed was stored in sealed plastic bags at 4°C until needed. Food consumption was determined daily for all animals. DON culture material and DON contaminated diets were handled using personal protective equipment. All reusable laboratory and feed mixing equipment were decontaminated by soaking in 10% hypochlorite before cleaning.

Exercise Stress

Animals were exercised by block and the order was determined randomly. The non-stressed controls were exposed to the noise of the treadmill by placing them in close proximity. Exercise occurred in the morning during the active nocturnal period. Animals were placed in individual lanes and exercised to fatigue by increasing the treadmill speed from 10 to a maximum of 28.5 m/min in 2-3 m increments every 20 min. Mice were removed from the treadmill when they could no longer maintain pace with physical prodding and returned to their cages and allowed access to food and water. Time to fatigue ranged from 2.25 to 3.5 hours.

Tissue Collection

After 30 minutes cage rest, exercised animals and a paired non-exercised control were euthanized by decapitation and trunk blood collected into microcentrifuge tubes containing 15 µl 10% EDTA, spleens were removed aseptically and immediately placed in centrifuge tubes containing RPMI 1640 supplemented with 50 µg/ml gentamicin, 2 mM L-glutamine,

10 mM HEPES buffer, and 10% heat-inactivated fetal bovine serum (complete media-CRPMI). Spleen weight was determined by difference of centrifuge tube weight before and after spleen collection. Splenocyte single cell suspensions were made by gently grinding spleens in fresh CRPMI between two frosted microscope slides; tissue fragments were settled out prior decanting. Spleen and trunk blood cell counts, Hb, and Hct were determined by automated cell counter (Hemavet®, CDC Technologies, Inc., Oxford CT) within two hours of collection. Remaining blood was centrifuged at 2500 rpm for 10 min, plasma separated and frozen at -20°C.

NK Cytotoxicity Assay

The spleen cell suspension was adjusted to 5 X 10^6 cells/ml with CRPMI and added to 96-well microtiter plates in triplicate for effector to target (E:T) ratios 25:1, 50:1, and 100:1. Preliminary studies on NK cytotoxicity response of BALB/c splenocytes to rhIL-2 (500 ng/ml) determined optimum activation concentration of 5 ng/well. rhIL-2 was added to each well (10 µl) except one set of 50:1 for unstimulated controls. CRPMI was added to equalized all wells at 220 µl. Plates were incubated for 20 hours at 37°C in 5% CO_2. The following day, 7.5 X 10^6 YAC-1 (ATCC), in log growth phase, were incubated with 200 µCi of ^{51}Cr (PerkinElmer, Boston MA) for 70 minutes. The target cells were washed three times in CRPMI, counted, diluted and 1 x 10^4 cells added per well. Six replicates of YAC-1 cells only with and without rhIL-2 were included each day. The plates were incubated for 4.5 hours at 37°C in 5% CO_2. YAC-1 cells in total release wells were lysed with the addition of 170 µl 10% trichloroacetic acid and plates were centrifuged at 500 rpm for 5 min. Supernatant (110 µl) was removed from each well by multichannel pipettor and transferred to microtubes. The tubes were loaded onto the gamma counter (Gamma Trac 1191) and each sample counted for one min. Mean ^{51}Cr release from YAC-1 controls, total release and splenocyte/YAC-1 combinations from each animal was calculated. Background control ^{51}Cr release and total release was used to calculate specific ^{51}Cr release at each E:T ratio for each animal's splenocyte sample. % Specific ^{51}Cr release = (Mean ^{51}Cr release per splenocyte sample– mean YAC-1 ^{51}Cr release)/ (Mean total ^{51}Cr release – Mean YAC-1 ^{51}Cr release) X 100. Percent YAC-1 ^{51}Cr release was calculated to verify target cell viability from equation: (Mean YAC-1 ^{51}Cr release - Mean media only blank)/ (Mean total ^{51}Cr release – Mean media

only blank) X 100. Background YAC-1 ^{51}Cr release from target cells was 4.43 ± 0.29% across the three days of sample collection.

Lymphocyte Proliferation

Spleen cell suspension (5 X 10^6 cell/ml) was pipetted (100 µl) into nine wells per animal in 96-well round bottom microtiter plates. Three replicate wells per animal were treated with: LPS 0.5 µg/well (10 µl), Con-A 1 µg/well (10 µl), and unstimulated controls with 10 µl media. Plates were incubated at 37°C, 5% CO_2 for 72 hours. MTS was added to all wells (10 µl) and incubated for 165 min at 37°C. Plates were read at 490 nm on a microplate reader (Benchmark™, Biorad Corp., Hercules CA). The difference between the mean mitogen stimulated wells and mean control wells for each subject was calculated and compared to the control treatment group to determine percent control response lymphocyte proliferation for each mitogen.

Hemolytic Plaque Assay

All mice were primed four days prior to sacrifice with 0.20 ml intraperitoneal injection of 20% sRBC (vol/vol) in PBS that had been washed three times with HBSS. The same batch of sRBCs was used throughout this study. Spleen cells were diluted with AIM-V® to 3 X 10^6 cells/ml. A 4% sRBC suspension in AIM-V® media was prepared the day of assay by washing sRBC three times with HBSS prior to dilution to final concentration. Guinea pig complement was absorbed to sRBC by rehydrating the lyophilized complement (Low-Tox®) with 1 ml Nanopure® (Barnstead Corp., Dubuque IA) water then mixing with 250 µl washed packed sRBC, incubating on ice for 10 min, centrifuging at 1500 rpm for 10 min and decanting the complement. This was repeated three times to remove alternate pathway components from the complement. Aliquots of the absorbed complement were stored at -20°C until day of use when it was thawed and diluted 1:3 with AIM-V® media within 20 min of use. The plaque assay was conducted using double-sided slide chambers (two microscope slides stuck together with double sided tape), according to the Cunningham and Szenberg (1968) protocol, constructed the day before the assay and verified to hold 50 µl per chamber. Equal volumes (50 µl) of the following components were mixed in 1.5 ml microcentrifuge tubes: mouse splenocyte suspension, 4% sRBC, and absorbed diluted guinea pig complement. Duplicates of each animal's splenocyte sample and negative control

samples using AIM-V® media instead of complement were prepared. Reaction mixtures (50 µl) were quickly pipetted into each side of one slide assembly resulting in three slide assemblies per animal, 4 test chambers and two no-complement control chambers. Slide edges were sealed with melted paraffin and incubated for 60 min at 37°C in humidified boxes. Plaques were counted by 4X magnification and multiplied by 20 to determine PFCs per 10^6 spleen cells. No plaques were observed in any of the negative control samples.

Cytokine Assay

Spleen suspension (5 X 10^6 cell/ml) was added to 24-well plates (1 ml/well) with stimulatory mitogens: Con-A (10 µg/ml media) for IL-2, IL-4, IL-10, and IFN-γ; LPS (5 µg/ml media) for IL-6; or media only. The plates were incubated at 37°C in 5% CO_2. Supernatant was removed from one well of each treatment at 24-hour intervals and transferred to cryotubes for storage at –20°C until assayed: IL-6 at 24 hours only; IL-2 at 24 and 48 hours; IL-4, IL-10 and IFN-γ at 48 and 72 hours of incubation.

Cytokine concentrations were determined using mouse monoclonal antibodies in kits (PharMingen, San Diego CA) and following manufacturer's instructions. Briefly, 96-well ELISA plates (Corning, Corning NY) were coated with cytokine specific capture monoclonal antibody overnight at 4°C. Plates were blocked with PBS-10% heat-inactivated fetal bovine serum for one hour at room temperature before the addition of standards and thawed supernatants (100 µl) and incubation at room temperature for two hours. Plates were washed between each step with PBS-0.05% Tween. Biotinylated anti-mouse cytokine monoclonal antibody with avidin-horseradish peroxidase conjugate was added to plates and incubated for one hour at room temperature. Plates were washed prior to addition of tetramethylbenzidine (TMB) and hydrogen peroxide substrate then incubated in the dark for 30 min and read at 655 nm on Biorad (Benchmark) microplate reader. Cytokine concentrations were determined from best fit linear regression of blanked mean absorbance of standards against standard concentrations.

Statistics

Two-way analysis of variance was conducted with SAS (SAS 9.1, Cary NC) using Tukey adjustment for multiple comparisons. Cytokine concentrations were compared by

repeated measures mixed analysis of variance procedure and Tukey-Kramer adjustment for multiple comparisons. P value of less than 0.05 was considered significant.

Results

All mice tolerated the diets well. The mice fed 2 mg DON/ kg diet gained significantly less weight over the 28 day feeding period compared with the other two diets while consuming significantly more feed daily (Figure 6.1).

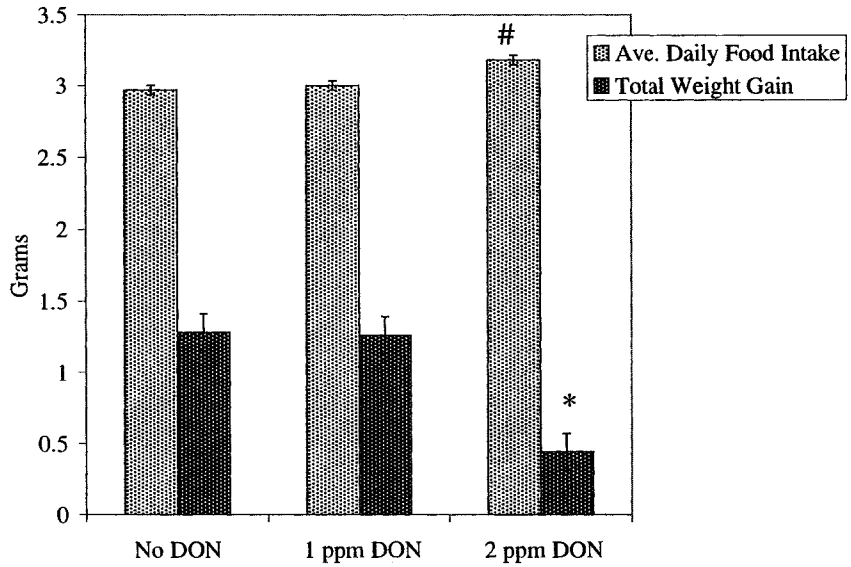

Figure 6.1. Reduced weight gain and increased feed intake in BALB/c mice fed 2 mg DON/ kg diet for 28 days. *Main effect of 2 mg/ kg dietary DON on weight gain over 28 day feeding period. Values are group mean ± SE, $p < 0.001$. n = 18 per dose. # Main effect of dietary DON, 2 mg/kg significantly different from 0 or 1 mg DON/ kg diet. Values are group mean ± SE, $p < 0.001$, n = 18 per dose.

Both DON and acute exercise stress had effects on white and red blood cells. Blood neutrophils were significantly increased by acute exercise stress (Figure 6.2). The neutrophil to lymphocyte ratio was also significantly increased by acute exercise (Figure 6.2). A significant interaction between DON and acute exercise ($p = 0.007$) occurred for circulating lymphocytes independent of DON dose (Figure 6.3). The effect of acute exercise on lymphocytes was greater, 42.5 ± 2.1% of control, than the effect of DON intake, 75.1± 4.8% of control.

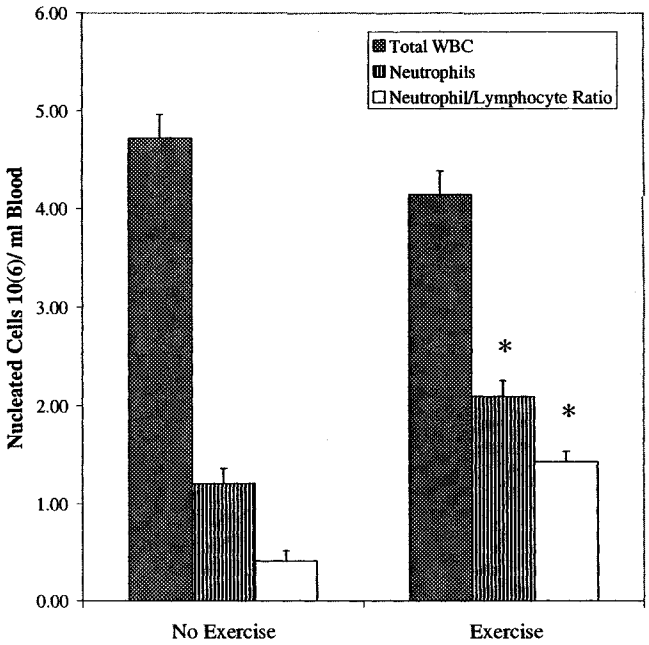

Figure 6.2. Blood neutrophils and neutrophil to lymphocyte ratio significantly increased by acute exercise stress. Trunk blood WBC differential counts from BALB/c mice fed 0, 1, or 2 mg/kg dietary DON for 28 days then exercised to fatigue on a treadmill. Total WBC was not different between exercised and non-exercised groups. *Significant main effect of acute exercise on neutrophil count, $p < 0.0003$, and neutrophil to lymphocyte ratio, $p < 0.0001$. Values are group mean ± SE, n = 27.

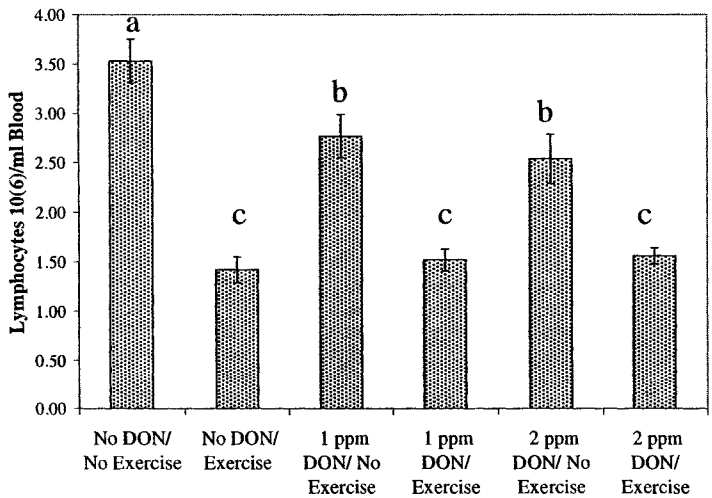

Figure 6.3. Acute exercise stress and dietary DON suppress blood lymphocytes. Trunk blood lymphocyte counts after 28 day feeding of 0, 1, or 2 mg DON/ kg diet and acute exercise to

fatigue on a treadmill. Different letters significantly different, $p < 0.05$. Values are group mean ± SE, n = 9.

Red blood cell parameters were affected by DON (Table 6.1) and exercise (Table 6.2) independently. Differences in sample sizes were due to loss of sample from blood clotting. Measured spleen related parameters also revealed significant effects due to treatment variables. Spleen cell count per weight of spleen was not affected by treatment.

Table 6.1. Red blood cell parameters suppressed by 2 mg/ kg dietary DON in BALB/c mice

	No DON	**DON 1 mg/ kg diet**	**DON 2 mg/ kg diet**
RBC count	9.4 ± 0.4	9.3 ± 0.3	8.0 ± 0.3*
10^9 cells/ ml blood	(n = 16)	(n = 17)	(n = 17)
Hematocrit %	46.1 ± 1.5	45.3 ± 1.3	39.6 ± 1.3*
	(n = 16)	(n = 16)	(n = 17)

Footnote. Blood collected at termination with EDTA anticoagulant and parameters analyzed by automated blood analyzer. Values mean ± sd of dietary groups with indicated animal numbers. *Significantly different from other two dietary groups, $p < 0.05$.

Table 6.2. Red blood cell and spleen weight affected by acute exercise stress in BALB/c mice.

	No Acute Exercise	**Acute Exercise Stress**
Hematocrit %	45.7 ± 1.1	41.7 ± 1.2*
	(n = 24)	(n = 26)
Hemoglobin	15.3 ± 0.2	14.2 ± 0.2*
(g/dl)	(n = 27)	(n = 27)
Spleen Weight (mg)	143 ± 7	118 ± 3*
	(n = 27)	(n = 27)
Spleen to Body	5.5 ± 0.1	4.9 ± 0.1*
Weight Ratio	(n = 27)	(n = 27)

Footnote. Blood collected at termination with EDTA anticoagulant and parameters analyzed by automated blood analyzer. Values mean ± sd of groups with indicated animal numbers. *Significantly different from non-stressed group, $p < 0.05$.

Functional immune assays were stimulated or unresponsive to treatments. Spleen cell suspensions were incubated with polyclonal T-cell mitogen Con-A and B-cell mitogen LPS. No significant treatment effects were observed for proliferation in response to either of these mitogenic agents. Splenocyte NK cytotoxicity was generally low but a main inhibitory effect of acute exercise stress occurred across all E:T ratios ($p < 0.05$) and a main effect of DON was observed with 1 mg/ kg being stimulatory compared to no DON or 2 mg/kg fed groups at the 100:1 E:T ratio (Figure 6.4). B-cells producing antibodies against sRBC were also stimulated by incorporation of 1 mg DON/ kg diet of BALB/c mice compared to 2 mg/ kg but was not significantly greater than non-DON fed groups (Figure 6.5).

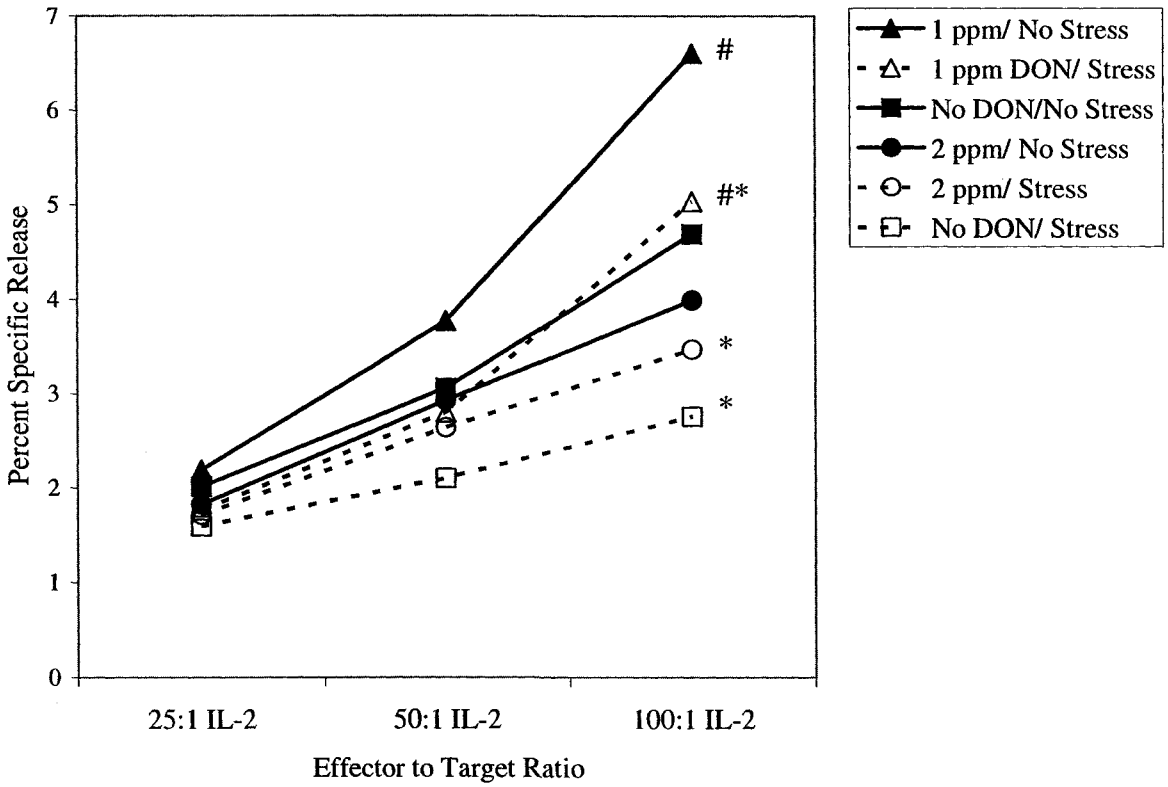

Figure 6.4. Natural killer cell function enhanced by 1 mg/ kg dietary DON and inhibited by acute exercise stress. Percent specific release measured by ^{51}Cr release assay. #Main effect of DON 1 mg/kg at 100:1 E: T ratio, $p < 0.01$. *Main effect of acute exercise stress in all rhIL-2 activated E: T ratios, $p < 0.05$. Values are group mean, SE not included for clarity, n = 9 per group.

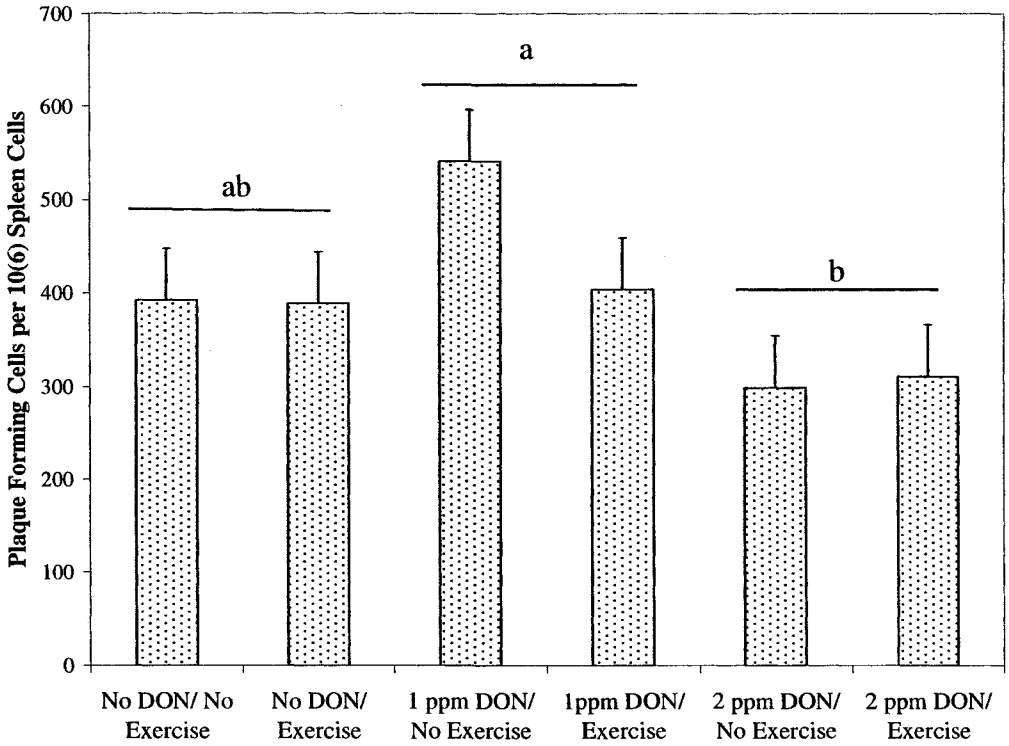

Figure 6.5. Plaque-forming cells specific for sRBC increased by 1 mg/kg dietary DON in BALB/c mice. Specific antibody production in spleen cells immunized with sRBC. All animals primed 4 days prior to termination, measured with hemolytic plaque assay. Main effect of 1 ppm dietary DON, different letters significantly different, $p = 0.0167$. Values are group mean ± SE, $n = 9$.

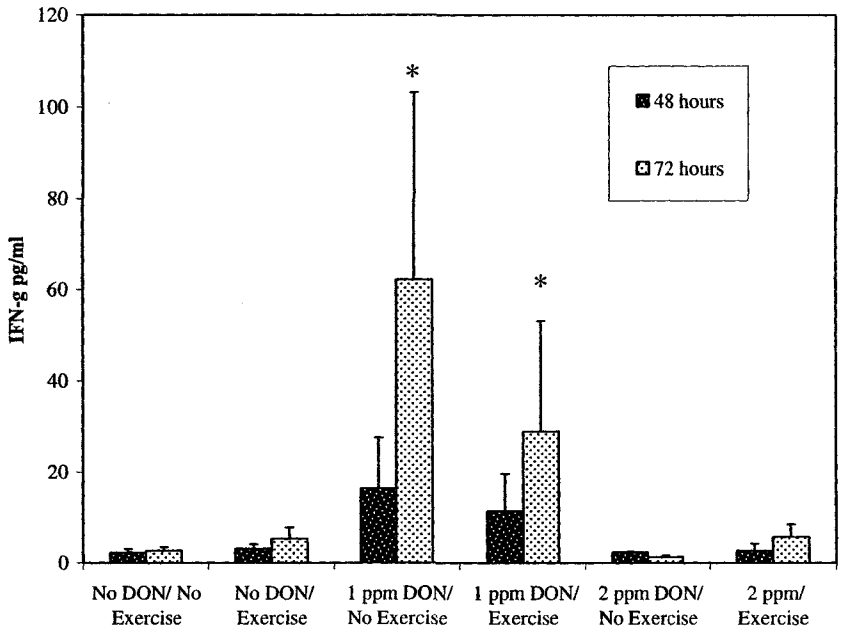

Figure 6.6. Increased spontaneous interferon-γ release from spleen cells of 1 mg/ kg dietary DON in BALB/c mice. ELISA measurement of cytokine release from spleen cell supernatants without mitogen stimulation at 48 and 72 hours of incubation. *Main effect of 1 mg DON/ kg diet at 72 hours incubation, $p \approx 0.02$. Values group mean ± SE, $n = 9$.

Of the five cytokines assessed from Con-A or LPS mitogen-stimulated splenocyte cultures, significant treatment effects were found only for IFN-γ and IL-4. No significant treatment effects were observed for IL-2, IL-6, or IL-10. Without mitogen activation, secretion of IFN-γ was significantly greater in the 1 mg DON/ kg diet groups after 72 h incubation (Figure 6.6) but was potently stimulated by Con-A across all treatment groups (1146.8 ± 71.0 pg/ml). There was strong significant correlation ($r = 0.79$, $p < 0.001$) between NK cytotoxicity and IFN-γ secretion. Con-A stimulated IL-4 secretion was significantly elevated in exercised mice not fed DON compared with unexercised controls and mice fed 1 mg/kg DON and not exercised. All other treatments did not differ statistically from either extreme in effect on IL-4 (Figure 6.7). Despite no significant treatment effects on spontaneous IL-2 release from spleen cell incubations, IL-2 supernatant concentrations were positively correlated to NK cytotoxicity ($r = 0.62$, $F = 39.29$, $p < 0.0001$) and PFCs ($r = 0.65$, $F = 37.61$, $p < 0.0001$).

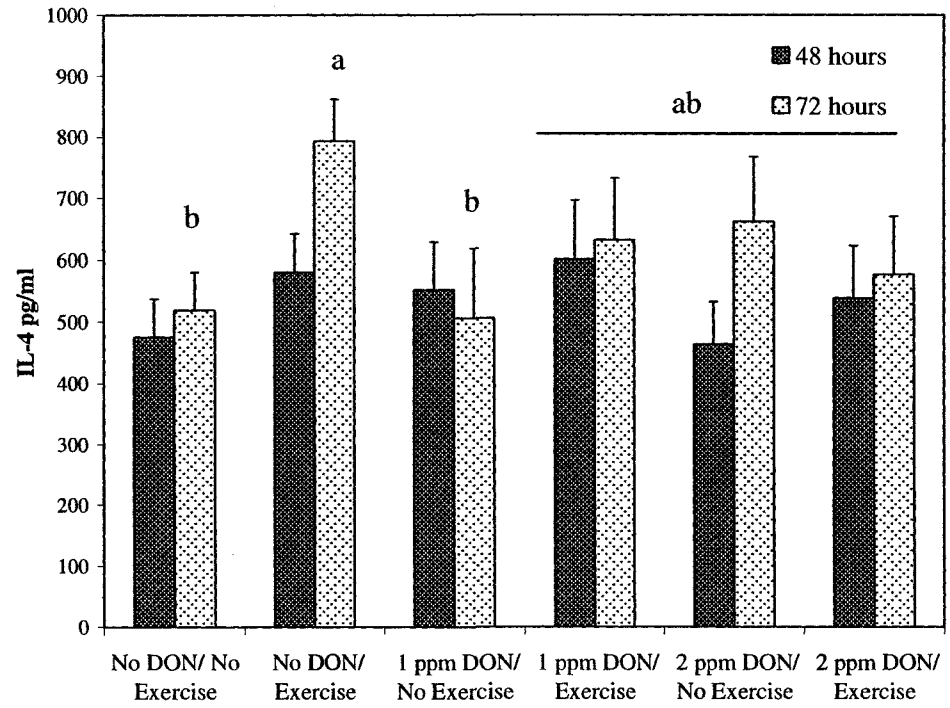

Figure 6.7. Stimulatory effect of acute exercise stress on IL-4 expression in Con-A stimulated murine spleen cells. ELISA measurement of cytokine release from spleen cells with Con-A (10 µg/ml) stimulation for 48 or 72 hour incubations. Different letters significantly different from each other, $p < 0.05$. Values group mean ± SE, n = 9.

Discussion

This study was designed to follow a standardized immunomodulation evaluation as proposed by Luster et al. (1988) and recommended by the National Toxicology Program (NTP, 2005). BALB/c mice fed 2 mg DON/ kg diet had reduced body weight gain similar to previous feeding study reports (Forsell et al., 1986, Greene et al., 1994). Sensitivity to DON feeding appeared comparable between B6C3F1 mice used in these studies and BALB/c mice. Increased feed intake has been reported in only one study feeding 20 mg DON/ kg diet to Sprague-Dawley male rats (Morrissey et al., 1985). 3-ADON was found in these samples but absence of other mycotoxins was confirmed by an outside laboratory (NDSU). Since culture material was used as the DON source, unknown appetite stimulatory compounds may have been present although this seems unlikely since only 40.4 g of culture material was added to each kg diet to achieve 2 mg/kg. 3-ADON did not significantly alter feed intake of weanling male mice fed 2.5 mg/ kg diet for 3 weeks (Kasali et al., 1985). Diet

form was a hard biscuit allowing easy daily monitoring whereas most diet studies use powdered diets which could be difficult to monitor subtle changes of intake.

Several changes in blood cells were observed due to exercise stress. Reduced circulating erythrocytes due to exercise stress may be related to oxidative damage to red blood cells. Oxidative stress occurred in acutely exercised mice (Azenabor and Hoffman-Goetz, 1999) and RBCs from BALB/c mice were more susceptible to H_2O_2 induced hemolysis than those from C57BL/6 mice indicating strain sensitivity (Kruckeberg, 1991). Even though total WBC counts were not different across treatments, significant neutrophilia was induced by acute exercise stress, also producing increased neutrophil: lymphocyte ratio. Men participating in a sprint triathalon had similar neutrophil and lymphocyte responses (Weinstock et al., 1997). Blood neutrophils from rats were increased in circulation due to shedding of endothelial L- selectin adhesion molecules within 30 minutes of stressful event, although causative agents were unidentified (Strausbaugh et al., 1999). Alterations of leukocyte numbers in circulation are due to differential cell trafficking.

Splenocyte activities were also affected by exercise stress. NK cell cytotoxicity inhibition following acute exercise stress was documented in humans (Pedersen et al. 1991) and mice (Randall Simpson and Hoffman-Goetz, 1990). The effect was transient, dependent on intensity and duration of exercise stress, and independent of training. Stress hormones were thought to mediate the effect by altering functional adhesiveness with the target cells and repressing the expression of granzyme A, granular killing protein, since NK cell numbers were not reduced (Zhou et al., 1997a). Other studies indicated reduced NK cell numbers following intense acute exercise due to the high number of β-adrenoceptors on NK cells resulting in redistribution in response to catecholamines (Pedersen and Hoffman-Goetz, 2000). Glucocorticoids were increased by acute exercise (Hoffman-Goetz et al., 1988; Landgren et al., 2005 manuscript in progress) although catecholamines were not measured. Reduced NK cell cytotoxicity may have been due to either of these mechanisms.

A significant reduction in Hct and RBC count was observed due to 2 mg/ kg dietary DON which may inhibit erythropoesis over the 28 day feeding period since RBC lifespan is 120 days. Arnold et al. (1986) found significantly reduced Hct in mice fed 6.25 mg/ kg DON for 5 weeks. No adverse effects on hemolysis or coagulation in mature human RBCs of

umbilical cord origin incubated with DON concentrations up to 2.96 μg/ml in medium (Froquet et al., 2003) suggesting the reduction in Hct and RBC count in our study was not due to direct effects of DON on mature RBCs.

Absolute blood lymphocyte count revealed an interaction between exercise stress and DON, with a dose independent suppression but a greater suppression across exercised groups. Reduction in circulating lymphocytes could occur due to DON induced apoptosis directly or in response to lack of survival signals or inhibited blastogenesis in the bone marrow. Since blood lymphocyte depression was not dose dependent, DON exposure at low doses may exhibit a threshold effect for lymphocyte precursors. Stress hormones were probably the main effectors of circulating lymphocytes after exercise stress (Weinstock et al., 1997) causing redistribution of lymphocytes from the circulation into lymph nodes, bone marrow and skin (Dhabhar, 2002). DON most likely acted by different mechanisms to affect lymphocytes, as we previously observed no corticosterone elevation from 2 mg/ kg dietary DON. Bone marrow and blood cell phenotyping may help to characterize lymphocyte responses to concurrent DON exposure and acute exercise stress.

NK cytotoxicity and PFC assays functionally assess innate and humoral immunity, respectively. This is the first report of significant *in vivo* immunostimulatory effects of DON on these two assays. 3-ADON fed to male CD-1 mice did not exhibit any inhibitory effects of mitogen stimulated lymphocyte proliferation or PFC response to sRBC at 2.5 μg/ g diet, the lowest dose tested (Tomar et al., 1987), therefore both of these effects may be explained by the effects of DON on cytokines. NK cells secrete IFN-γ when activated in response to IL-2. IL-2, produced by T-cells in response to IL-1 and IL-12 from macrophages, stimulates B-cell differentiation and autocrine activation of T-cells (Abbas et al., 1996). Despite no significant treatment effects on spontaneous IL-2 release, IL-2 concentration had strong positive correlation with NK cytotoxicity. *In vitro* DON exposure caused increased IL-2 and sustained production for 120 hours (50 and 100 ng DON/ml in media) in murine EL-4 thymoma cells but was inhibited 500 ng DON/ ml (Li et al., 1997). Miller and Atkinson (1986) reported that DON (0.005 to 0.5 ng/ml) stimulated rat blood lymphocyte proliferation and stimulatory factor(s) were secreted into supernatant from DON-exposed macrophages. IL-12 was a potent regulatory cytokine produced by macrophages and dendritic cells which

stimulated NK cells to produce IFN-γ (Elenkov and Chrousos, 2002). Induced expression of IL-12 mRNA has occurred with acute dosing of DON to mice (Zhou et al., 1997b). We propose a low-dose stimulatory effect of DON on macrophages that led to IL-12 release, T-cell IL-2 release that activated NK cells and promoted differentiation of antigen-specific B-cells. This dose response may fit the description of hormesis, low dose stimulation and high dose inhibitory effects.

In an extensive literature review of immunologic hormetic-like responses, Calabrese (2005) listed a large number of endogenous substances, pharmacologic agents, and toxicants that stimulated one or more immune endpoints at low doses and exhibited immunotoxic effects at higher doses. BALB/c mice were reported to be the second most common animal model with hormetic-like biphasic dose responses. Whether the immunostimulation was beneficial or harmful may depend on the antigenic challenge(s), confounding or interactive immunomodulators. Mechanisms for the immunostimulatory effects observed in our study most likely were due to complicated and not fully defined DON effects on cellular signal transduction pathways. Shifrin and Anderson (1999) determined that trichothecene protein synthesis inhibitors bound to the ribosome and triggered stress signal pathways termed the "ribotoxic stress response" not necessarily requiring translational arrest. *In vitro* DON exposure activated or inhibited mitogen-activated protein kinase (MAPK) pathways depending on cell type, dose, and length of exposure and immune system effects could occur as MAPK pathway activation can stimulate expression of cytokines or activate apoptosis (reviewed by Pestka et al., 2004). *In vivo* DON induces MAPKs but studies have been limited to high acute doses. Other molecules upstream or downstream of MAPK may be identified to have inhibitory or stimulatory effects on cellular function.

Oxidative stress mechanisms may also play a role in DON toxicity. Redox homeostasis is maintained in the cell mainly by glutathione but the buffering system can be overwhelmed if key enzymes in the synthesis of reactive oxygen species (ROS) scavenging molecules are inhibited (reviewed by Morel and Barouki, 1999). Ueno and Matsumoto (1975) determined that fusarenon-X, a chemically related trichothecene with an acetyl substitution for the hydroxyl of DON at C3, significantly inactivated three different thiol-enzymes when incubated prior to addition of the specific enzyme substrate, one mycotoxin

molecule bound in each critical –SH. High affinity protein synthesis inhibition by DON is conceivable, particularly for proteins that are constitutively synthesized. No other work on this effect of trichothecenes has been reported nor discussed as a toxic effect. Numerous molecules in signal transduction pathways rely on active thiol motifs, particularly in reactive oxygen species homeostasis. DON at low doses may promote chronic oxidative stress by binding to constitutive ROS scavenger enzymes resulting in immune function dysregulation.

Immunostimulatory and inhibitory functions of the immune system of BALB/c mice were identified following 28 day feeding of two doses of DON with an acute exercise stress co-variable. This is the first report, to our knowledge, of functional *in vivo* immunostimulatory responses of 1 mg/ kg dietary DON and hormetic-like dose responses. Mechanistic studies to identify effects of DON on immune-related gene expression and especially DON-sensitive proteins, such as cytokines and free radical scavengers, in global assays, would further characterize the complicated signal transduction effects observed to be affected by DON. Investigations of DON toxicity using human lymphocyte proliferation assays should control for possible prior low-dose DON or other immunotoxicant exposure due to the possibility of confounding suppression of circulating lymphocytes. In the controlled conditions of animal feeding trials, complicated *in vivo* effects are not easily explained. Dietary relevance of these doses to human exposures should be a key consideration. Further studies are warranted to investigate length of exposure effects, differential effects on immune cell subsets, and ultimately host resistance effects.

CHAPTER 7. GENERAL CONCLUSIONS

General Discussion

This body of work covered a wide range of disciplines while investigating one mycotoxin, deoxynivalenol. Interest in this mycotoxin seemed warranted as it was ubiquitous in human food supplies, stable to all but alkaline food production processes, substantial but ineffective efforts to prevent cereal plant infection with DON-producing *Fusarium* fungi, and toxicological effects in monogastric species at DON doses similar to frequently occurring food supply levels.

In order to conduct any analytical or screening method development, a reference method either in house or in a referral laboratory is required. HPLC was available in our laboratory but no internal standard addition methods had been published therefore established HPLC methods required a DON spiked sample along with the unknown to quantify the analyte. Caffeine proved to be a suitable internal standard due to its stability in the solvent systems, compatible UV absorbance, and absence from analytical samples of interest. Once a standard method was established, screening methods could be developed for comparison. As our knowledge of DON toxicity grew it became apparent that the immune system was particularly sensitive to DON exposure and development of a bioassay based on immune cell models would serve two purposes. K-562 cells were chosen as a model because they were one of the most sensitive cells to cytotoxicity from DON exposure which was comparable to human peripheral lymphocytes. Thus the K-562 assay could be used as a model for a known biological effect while being sensitive enough to screen cereal samples and accurately predict DON concentrations compared to HPLC analysis.

The body of evidence investigating low dietary exposures (less than 10 ppm) to DON has revealed variable immunologic effects, unresponsive to immunosuppressive in numerous mouse strains. Impaired host resistance in mice and anecdotally in humans indicate the potential of effects. We hypothesized that immunotoxic effects of DON may be potentiated by an additional insult on the immune system, acute exercise stress. Two animal trials investigating this effect found supporting evidence for interaction between these two immunomodulating variables. The first study found suppressed PFCs to sRBC after two weeks of 2 ppm and a possible antagonistic but beneficial effect on lymphocyte proliferation

suppression. The second study did not support these finding for 2 ppm dietary DON after 28 d feeding but interestingly found immunostimulation of NK cytotoxicity and PFCs due to 1 ppm dietary DON which was slightly modulated by acute exercise stress. Both of these immune function assays measure *ex vivo* responses and together have shown high ability to predict true *in vivo* immunologic effects. The two animal studies differed in enough variables (age of animals tested, length of time of DON exposure, and source of dietary DON) that the studies were considered independent. The most likely reason for the apparent differences between the studies is the length of feeding as animals have shown adaptation to DON exposure over similar time periods. We hypothesized that 1 ppm dietary DON would be below the threshold for immunological effects of DON and were surprised by the stimulatory effects observed. Based on the cytokines measured, antagonistic effects between dietary DON and acute exercise stress seemed to act through independent mechanisms on lymphocyte subsets and cytokine production altering the balance between Th1/Th2 immune responses.

Recommendations for Future Research

This line of research could develop following numerous pathways. Initially the biological significance of immunostimulation should be investigated. Functionality of both the cellular and humoral arms of the immune system should be challenged as stimulation was observed in both i.e. Herpes simplex virus or tumor challenge and *Listeria* or *Salmonella* challenges. Investigations should attempt to define the length of exposure effect as it seems two different responses may be observed with this variable alone. Additional dose response studies are also warranted investigating lower dietary doses to define the dose-response curve. Additional insight into the mechanism of effect may be defined by broadening the battery of cytokines measured to include IL-12, IL-1, and any new cytokines for which reagents are available with possible involvement in the cellular effects observed. Concurrent with the challenge studies, immune cell populations and subsets should be investigated with specific marker phenotyping. This could be directly correlated with cytokine expression observed and has the advantages of being relatively easy to perform and directly reflects *in vivo* effects. Potentially more specific mechanistic information could be obtained from global investigation techniques becoming more popular. DON is an established protein synthesis

inhibitor although little is known about specificity or sensitivity to constitutively expressed proteins. The challenge to this approach will be development of the profile of proteins to be investigated. An ongoing line of research has focused on mRNA expression in response to DON exposure but known effects of DON are on transcription and cell signaling pathways questioning the utility of genomic investigations. Proteomic and/or metabolomic investigations may reveal high affinity protein alterations that may further characterize the molecular mechanisms of DON's *in vivo* immunomodulatory effects.

Ultimately the worth of any of this research will only be validated if epidemiologic studies are conducted relating DON exposure in humans with risk of disease. Human illness has occurred concomitant with epidemics of moldy grain but definitive evidence of correlation has not been made. The ultimate question that deserves an answer: Does human exposure to dietary DON increase risk of disease with or without confounding immunomodulatory factors i.e. environmental stressors, nutritional deficiencies?

APPENDIX – HPLC ANALYSIS OF MURINE URINE AND FECAL SAMPLES

Metabolic Cage Set-up for Urine and Feces Collection

After the animals had been on treatment diets for at least 2 weeks, animals were placed in metabolic cages by random selection of the block order. This block order was maintained through the remainder of the experiment. Rat Nalgene® metabolic cages were modified to accommodate the comfort of the mice and most accurately collect urine and feces. A small gauge wire screen (3/8 inch chicken wire) was inserted over the floor of the cage. The rat food chute was not used. Alternatively the food biscuits were attached to a screen square placed outside of and over the food chute opening and close approximation was maintained with long rubber bands around the upper portion of the cage, therefore the mice ate their food through the squares of the grid and diet contamination of excretion was minimized. To increase recovery of urine and feces, the surface of the collection cone inside the cage was lightly coated with food-grade non-stick spray, urine was collected directly into a plastic 15 ml centrifuge tube and the animals were held in the metabolic cages for 48 hours. The inside of the collection funnel was rinsed with about 5 ml of 20% aqueous methanol to increase the recovery of urine. Fecal pellets were collected into a separate test tube with screw-top. Any large pieces of food that may have fallen into the collection tube were removed prior to storage of all samples at -20°C until analysis. Animals were rested in their shoebox cages for at least three days prior to any other procedure.

Urine Analysis for Deoxynivalenol

One the day of analysis, urine samples were thawed and divided in half into two 25 or 50 ml flasks. An equal volume of acetate buffer (250 ml Milli-Q water, 5.78g sodium acetate, and 0.45 ml glacial acetic acid) was added to each sample. In one replicate, 5000 U β-glucuronidase was added and all flasks were sealed with Parafilm. Flasks were incubated overnight at 37°C on shaker water bath.

Flasks were removed and allowed to cool and a stir bar was added. A volume of acetonitrile was added in the ratio 5.25 ml per ml of urine-buffer solution. The ACN was added slowly while the mixture was stirred and continued for 10 minutes. Solid phase extraction was accomplished by putting entire sample through a 5 g 20:1 alumina – charcoal column. Eluate was collected in a 125 ml round bottom flask. Do not allow the SPE packing

to go dry between extract additions. When last addition reaches the top of the packing, rinse the incubation flask with 5 ml of 84:16 aqueous ACN and add to column then allow to drain completely. Evaporate contents of round bottom flask on Rota-vap, with 50°C water bath, until about 1 ml residue left. Transfer to 12 X 75 cuvette with Pasteur pipette. Rinse flask twice with 1 ml DI water and add to cuvette.

Precondition Sep-Pak C18 columns with 5 ml 100% methanol then 5 ml Milli-Q water. Load sample onto preconditioned column. Rinse Sep-Pak with 2 ml water, blow out residue water. Elute DON with 1 ml 40% aqueous methanol into a 12 X 75 cuvette. Evaporate sample completely under gently stream of nitrogen in 50°C water bath. Dissolve residue with 200 µl 20% aqueous methanol, briefly vortex, and filter through 0.45 µm syringe filter into plasma insert in HPLC autosampler vial.

Samples (50 µl injection) were analyzed by HPLC/UV with flow rate of 0.7 ml/min. The following gradient was used: mobile phase 10% methanol, 90% buffered (pH ~7.0) water, linear gradient to 30 minutes 10 to 15% methanol, 5 minutes 15 to 80% methanol, 3 min at 80% methanol, 2 min to return to 10% methanol, then 15 min at 10% methanol for equilibration, total run time 55 minutes.

Fecal analysis for Deoxynivalenol

Fecal samples were dried at least 24 hours in exhaust hood prior to weighing and division of sample in half into two glass screw-top test tubes. Tubes were sealed with aluminum foil and samples were autoclaved for 5 min at 121°C, allow cooling in hood. Remove foil and add 2 ml of acetate buffer, sit on bench for 3-4 hours to soften fecal pellets. Samples were vortexed periodically to facilitate homogenization. Add 50 µl β-glucuronidase (5000 U) to one sample replicate. Incubate all samples overnight in 37°C water bath.

Transfer sample to 25 or 50 ml flask with magnetic stir bar, rinse tube with 1 ml acetate buffer, and stir sample for 10 min to break up any remaining fecal pellets. Add 15.75 ml ACN slowly to sample while stirring and continue to mix for 30 min to extract DON. Run all of sample through SPE column of 2.5 g 40:1 alumina – charcoal packing in 12 ml syringe with #4 Whatman filter frit at bottom of syringe and ball of glass wool at top of packing. Collect eluate into large (20 ml) test tube. Rinse flask twice with 2 ml 84:16 aqueous ACN and add to SPE column. Drain completely then dry until about 1 ml residual extract under

gently stream of nitrogen in 50°C water bath. Add 1 ml water to sample tubes and vortex. Transfer sample by Pasteur pipette to open barrel of 3 cc syringe attached to preconditioned (as for urine) Sep-Pak C18 column. Rinse sample tube with 1 ml water and add to syringe before inserting the plunger and loading the sample on the Sep-Pak. Wash column with 2 ml water then elute DON with 2 ml 40% aqueous methanol into a 12 X 75 cuvette. Dry samples completely under gentle stream nitrogen in warm water bath. Dissolve residue with 150 µl 20% aqueous methanol and filter into plasma inserts in HPLC autosampler vials.

Samples were analyzed by HPLC/UV with 50 µl injection with 1 ml/ min flow rate. Linear gradient with 6% methanol 94% buffered Milli-Q water as mobile phase, gradient to 15% methanol in 40 min, step-up to 80% methanol in 5 min, hold at 80% for 3 min, equilibrate back to 6% methanol for 15 min with total run time per sample of 65 min.

Results

Numerous samples were utilized to optimize the analysis. Urine and feces from animals not fed DON were used for spiking samples and recovery. The following table gives data on DON intake and excretion from animals in the second mouse study, as available.

DON Intake and Excretion in BALB/c Mice fed Dietary DON Subacutely

Urine										
Mouse#	DON level	24h feed intake (g)	24h DON intake (ng)	24h 3-ADON intake (ng)	Free DON (ng)	Total DON (ng)	% DON intake as free DON	% DON intake as total DON	% total DON + 3-ADON intake	% excreted as conjugate
5	2 ppm	3.28	6510	5930	5161	7549	79.3	115.9	60.7	31.6
13	2ppm	3.34	6630	6040	2348	2042	35.4	30.8	16.1	0
17	2 ppm	2.82	5600	5100	3761	2867	67.2	51.2	26.8	0
22	2 ppm	3.54	7030	6400	2803	3079	39.9	43.8	22.9	8.9
29	2 ppm	2.9	5760	5240	7459	8963	129.5	155.6	81.5	16.8
Mean							70.3	79.5	41.6	11.5
sd							37.9	53.8	28.2	13.3
33	1 ppm	3.37	3340	3040	1356	2895	40.6	86.7	45.4	53.1
50	1 ppm	3.3	3270	2980	1042	5488	31.9	167.8	87.8	81
55	1 ppm	2.81	2790	2540	374	811	13.4	29.1	15.2	53.8
Mean							28.6	94.5	49.5	62.6
sd							13.9	69.7	36.5	15.9
Fecal										
2	2 ppm	3.06	6080	5530	225	282	3.7	4.6	2.4	20.3
7	2 ppm	3.55	7050	6420	275	236	3.9	3.3	1.7	0
8	2 ppm	2.77	5500	5010	614	697	11.2	12.7	6.6	12
9	2 ppm	3.39	6730	6130	245	234	3.6	3.5	1.9	0
20	2 ppm	3.21	6370	5800	415	265	6.5	4.2	2.2	0
34	2 ppm	2.89	5740	5230	205	324	3.6	5.7	3	36.8
38	2 ppm	2.95	5860	5330	238	313	4.1	5.3	2.8	23.9
41	2 ppm	2.94	5840	5320	569	584	9.7	10	5.2	2.6
44	2 ppm	2.94	5840	5320	364	320	6.2	5.5	2.9	0
Mean							5.8	6.1	3.2	10.6
sd							2.9	3.2	1.6	13.6
1	1 ppm	2.93	2910	2650	355	283	12.2	9.7	5	0
4	1 ppm	3.52	3490	3180	244	151	7	4.3	2.2	0
6	1 ppm	2.76	2740	2490	219	667	8	24.3	12.7	67.2
30	1 ppm	2.71	2690	2450	294	232	10.9	8.6	4.5	0
43	1 ppm	3.23	3200	2920	63	352	2	11	5.8	82.3
56	1 ppm	2.68	2660	2420	74	221	2.8	8.3	4.4	66.5
Mean							7.2	11	5.8	36
sd							4.1	6.9	3.6	39.8

From this preliminary analysis of DON excreted in urine and feces from mice fed two dietary levels, the majority of DON was excreted in the urine while less than 10% was

excreted in the feces. No animals had data for both urine and feces therefore the total excretion could not be evaluated. Glucuronide conjugation did occur in these animals with interindividual variablilty. The inclusion of 3-ADON in the diet appeared to contribute to free and total DON excretion in some animals but not in others. From this data it was apparent mice excreted the majority of ingested DON through the urine and glucuronide conjugation occurred by variable extents across individuals. These animals were on test diets for at least 14 days prior to excrement collection so steady state intake and excretion should have been reached. Purified or confirmed DON only feeding would further define the metabolic pathway of DON.

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