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DEVELOPMENT OF A HYBRIDOMA SECRETING MONOCLONAL ANTIBODY TO CITRININ

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

John Eric Line

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ABSTRACT

Citrinin is a highly potent nephrotoxin produced as a secondary metabolite during the growth of numerous fungi. Though citrinin may be detected in a variety of food products, it is most commonly found as a contaminant of both the large and small grains. It has been implicated in the production of renal disease and death among livestock and is implicated in a fatal renal disease in humans.

Current methods used to detect citrinin such as high performance liquid chromatography (HPLC) and thin layer chromatography are either expensive, require extensive research equipment or are not satisfactory for the detection of the small, but toxic, levels of mycotoxin which may be present in a food, feed or blood sample. The objective of this study was to develop a hybridoma producing monoclonal antibody to citrinin for use in an enzyme-linked immunosorbent assay (ELISA) for the mycotoxin.

Citrinin was coupled to bovine serum albumin (BSA). Analysis of buffers by HPLC following equilibrium dialysis revealed a molar binding ratio of 19 moles of citrinin per mole BSA. This citrinin/BSA conjugate was used to immunize 8-week-old male RBF

mice over a period of 6-8 weeks. Analysis of sera from the immunized mice by indirect ELISA showed that citrinin specific antibody was formed to the immunogen. Spleen cells from the immunized mice were fused to FOX-NY myeloma cells in the presence of polyethylene glycol. Actively growing hybridomas were expanded and screened by ELISA. Hybridomas positive for the production of anti-citrinin antibody were cloned by a limiting dilution technique and the clones then expanded and rescreened by ELISA. Positive clones were expanded and stored in liquid nitrogen as appropriate. It is anticipated that a monoclonal antibody developed by this method may be used in the rapid screening of agricultural commodities or in diagnosis of animals suspected to be suffering from citrinin toxicosis.

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INTRODUCTION

1. STATEMENT OF PROBLEM

Citrinin is a highly potent nephrotoxin produced as a secondary metabolite during the growth of numerous fungi. Though citrinin may be detected in a variety of food products, it is most commonly found as a contaminant of both the large and small grains. It has been implicated in the production of renal disease and death among livestock and is implicated in a fatal renal disease in humans.

Current methods used to detect citrinin such as high performance liquid chromatography (HPLC) and thin layer chromatograpy are either expensive, require extensive research equipment or are not satisfactory for the detection of the small, but toxic, levels of mycotoxin which may be present in a food, feed or blood sample. Within the past decade methods have been developed to produce monoclonal antibodies (MABS) against many mycotoxins for subsequent use in an enzyme-linked immunosorbent assay (ELISA) or radio-immuno assay (RIA) for detection of the toxins.

Once developed, these tests are not only inexpensive and easily performed, but are among the most sensitive of detection methods available.

2. OBJECTIVES

The objectives of this study were to:

a) conjugate the low molecular weight citrinin to a higher molecular weight protein carrier, bovine serum albumin (BSA), in order to produce a suitable antigen for immunization purposes and to determine the molar binding ratio of citrinin to BSA;

b) develop an ELISA technique for analysis of antibody produced against citrinin and;

c) develop a hybridoma producing monoclonal antibody to citrinin.

It is anticipated that this monoclonal antibody may be used in the rapid screening of agricultural commodities or in diagnosis of animals suspected to be suffering from citrinin toxicosis.

3. MYCOTOXINS

Justifiable concern exists regarding the carcinogenicity, mutogenicity and teratogenicity of fungal metabolites. Toxic fungal metabolites are generally referred to as mycotoxins and have been

implicated as the causative agents of death and disease among man and his animals throughout recorded history.

Today, statistics indicate that mycotoxins still pose a serious health problem to both humans and animals. In areas of Southeast Asia and Africa where contamination of food crops by aflatoxins is common, the incidence of human liver cancer is high. A fatal human renal disease called endemic Balkan nephropathy occurs primarily in areas of the Danube River basin of Yugoslavia, Bulgaria and Romania. Frequent contamination of food by citrinin and ochratoxin in this area has been implicated. Mycotoxins are economically devastating as well. In 1977, aflatoxin contamination of corn in the southeastern United States cost farmers an estimated $111 million. Mycotoxins are a problem not only in the United States but worldwide. Mycotoxins pose an especially serious problem in the Third World Countries where efforts to maintain export markets may lead to the consumption of poorer quality materials by the indigenous population resulting in increased exposure to mycotoxins (Smith and Moss, 1985).

Mycotoxins comprise a very diverse group of chemical compounds: they are produced by a taxonomically wide range of filamentous fungi and show

a diverse range of toxic effects (Smith and Moss, 1985). The four primary classes of mycotoxins include the aflatoxins, ochratoxins, zearalenone and tricothescenes (World Health Organization, 1979). One mycotoxin which has received little notoriety, though it is certainly worthy of further study, is citrinin.

4. CITRININ

Citrinin is produced by no less than 14 species of Penicillium, 3 species of Aspergillus and from the leaves of an Australian plant, Crotalaria crispata (Figure 1). Citrinin was first isolated from Penicillium citrinum in 1931 and was hailed as an antibiotic effective against Gram-positive bacteria until the discovery of its nephrotoxic activity prohibited its use as a therapeutic agent (Bureau of Veterinary Medicine, 1980; Betina, 1984). Citrinin-producing fungi are distributed worldwide and have been isolated from a variety of foods and feeds. Samples of corn, wheat, barley, rye, peanuts, oats, rice and apples have all been implicated as substrates for fungi growth and subsequent citrinin production (Betina, 1984).

Genus *Penicillium*

P. citrinum
P. fellutanum
P. lividum
P. implicatum
P. jenseni
P. velutinum
P. canescens
P. viridicatum
P. palitans
P. claviforme
P. citreo-viride
P. streckii
P. notatum
P. expansum

Genus *Aspergillus*

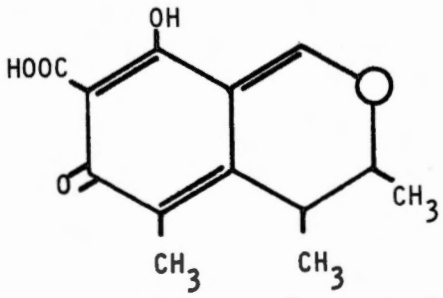
A. terreus
A. niveus
A. candidus

Source: Wyllie, T.D., and L. G. Morehouse (ed.) 1977. Mycotoxic fungi, mycotoxins, mycotoxicoses: an encyclopedic handbook. Marcel Dekker, Inc., New York, N.Y.

Figure 1. Species of fungi reported as producers of citrinin.

Citrinin, [(3R-trans)-4,6-Dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid], has a molecular weight of 250.0841 and forms lemon yellow needles when crystallized from alcohol (Figure 2). Citrinin is soluble in hot ethanol, ethyl acetate, benzene, acetone and chloroform. It is slightly soluble in diethyl ether, light petroleum ether, cold ethanol and hot water (Betina, 1984). Citrinin has a melting point of 178-179°C. Ultraviolet absorption for citrinin in 95% ethanol occurs at 319 (E=4710), 253 (E=8279) and 222 (E=22,280) nm (Wyllie and Morehouse, 1977). The fluorescence excitation maximum in 95% ethanol is 336 nm and the emission maximum is 521 nm. Citrinin exhibits a bright yellow fluorescence under longwave ultraviolet light.

The toxicity of citrinin has been observed in a wide variety of animal species including mice, rats, hampsters, rabbits, dogs, pigs, chickens, and guinea pigs. The greatest pathological change observed in all species exposed to citrinin is kidney damage. The mycotoxin produces "degeneration and necrosis of the tubular portion of the nephron. Grossly, the kidneys are swollen, pale-tan, and may have pale foci or radial streaks in the cortex. The portion of the tubular nephron most severely affected varies somewhat



Source: Betina, V. 1984. Mycotoxins - production, isolation, separation and purification. Elsevier Science Publishers, Amsterdam, Netherlands. 217-236.

Figure 2. Structure of citrinin.

with species and dose, but commonly damaged portions in most species are the straight segments of the proximal and distal tubules." (Bureau of Veterinary Medicine, 1980). The experimental LD_{50} varies greatly between species and among researchers. The LD_{50}'s for mice determined by intraperitoneal injection has been reported between 5 mg/kg and 52 mg/kg body weight (Wyllie and Morehouse, 1977). For rabbits, the LD_{50} was found to be 19 mg/kg body weight by intravenous injection (Betina, 1984). The determination of LD_{50} values is made difficult by the fact that citrinin may cause delayed deaths.

Citrinin is implicated in renal disease and death among livestock, poultry, and possibly humans. As mentioned previously, citrinin and ochratoxin are thought to be co-causative factors in endemic Balkan nephropathy. This disease is most common in individuals between 30 and 50 years of age and progresses slowly up to death. The victims' kidneys are reduced in size and are characterized by tubular degeneration, interstitial fibrosis, and hyalinization of glomeruli in the more superficial part of the cortex (World Health Organization, 1979). Many similarities in the structural and functional degeneration of the kidney have been noted between endemic Balkan

nephropathy and ochratoxin/citrinin induced porcine nephropathy, suggesting a common causal relationship.

Citrinin, by itself, is not thought to be carcinogenic though it may promote renal carcinogenesis when in conjunction with other potent carcinogens. While studies show that citrinin is not teratogenic in the mouse, it has proven to be teratogenic in chicken embryos (Bureau of Veterinary Medicine, 1980). Citrinin appears to be mutagenic to certain microbial systems yet is has no affect on others.

Relatively few studies have been done concerning citrinin, primarily because methods for detecting the toxin have been poor. The earliest and most common technique for citrinin determination is thin layer chromatography (TLC). Though this technique is fairly simple and inexpensive, it is not sensitive enough to detect very small amounts of citrinin, nor is it suitable for examining blood or urine samples. The most sensitive technique found thus far requires a high performance liquid chromatograph (HPLC) for analysis of contaminated samples. This procedure can detect as little as 2 to 5 ng of citrinin and is suitable for examining blood or urine samples (Marti et al., 1978; Phillips et al., 1980). Common use for citrinin

detection is prohibited, however, because of the cost of the instrument, solvents and columns.

The need exists for a cost efficient method for detecting citrinin which is sensitive enough to recognize the small yet harmful amounts of citrinin that may be present in a food, feed or blood sample. Such a technique may be made possible by utilizing a new biotechnological tool called a monoclonal antibody.

5. MONOCLONAL ANTIBODIES

Monoclonal antibodies are valuable because they rely on the intricacies of the immune system to generate the components for a test more sensitive than most mechanical systems possible. In 1975, Georges Kohler and Cesar Milstein reported the first successful fusion of a myeloma cell line with spleen cells from an immunized mouse. The result was a hybrid cell line which could live indefinitely in culture and secrete antibodies of a predetermined specificity (Kohler and Milstein, 1975). This technique has enabled scientists to develop diagnostic tests which rely on the great specificity of these antibodies to determine the presence or absence of a specific antigen. Levels of substances in the 0.2 ppb range are commonly detected by assays involving MABS. This

technique has revolutionized many areas of research and earned Kohler and Milstein a Nobel prize in 1984.

When an animal is injected or otherwise infected with a foreign protein three basic types of cells respond, the phagocytes and two kinds of lymphocytes - the T cells and B cells. Each has its own stratagies of defense. The first cells to respond would be the phagocytes. A special type of phagocyte called a macrophage can engulf the foreign protein. After injesting the foreign protein, called an antigen, the macrophage alerts the T cells. The body produces T cells by the tens of millions and some of the T cells will be specific for the antigen presented by the macrophage. Some of the T cells will attack the foreign protein directly, others will alert the B cells which will begin producing antibodies that are specifically targeted toward the antigen. The antibodies are so specific that they fit the antigen like a lock and key. The antibodies selectively bind to the antigens making it easier for the macrophages to engulf the invading foreign matter.

The principles of monoclonal antibody production are elegant. Through their application the specific synthesis of a single antibody-forming cell can be captured and "immortalized" in tissue culture (Golub,

1987). When antibody secreting cells from the bone marrow, thymus, lymph nodes or spleen are cultured in vitro, they have a relatively short lifespan. Myeloma cells, tumors of B-cells, while they may not secrete antibody, have an infinite lifespan in culture. By fusing antibody secreting spleen cells of a mouse with myeloma cells, Kohler and Milstein succeeded in producing hybrid cells which could secrete antibody and live indefinitely in culture. The hybrid created upon fusion of the two cell types in the presence of a variety of agents, normally polyethylene glycol (PEG), is refered to as a hybridoma.

Following fusion it is necessary to separate the fused hybridoma cells from the normal, unfused spleen cell population. This is easily accomplished because the unfused spleen cells die off in culture after only a short time. This leaves only the hybridoma cells and any remaining unfused myeloma cells, both of which are immortal. A method is therefore required to remove the unfused myeloma cells. This is done by utilizing myeloma cells that are killed in the presence of the drug aminopterin.

The usage of mutant myeloma cells to select fused hybridomas is perhaps best described by Edward Golub in his book, Immunology: A Synthesis (1987).

Myeloma cells, like most cells, use two pathways of DNA synthesis: the major synthetic pathway and a salvage pathway. Normal cells synthesize DNA using both pathways. When the drug 8-azaguanine is added to normal cells, it is incorporated into DNA through a reaction catalyzed by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) via the salvage pathway. Such cells die because they cannot funcion with the altered base. A variant cell that cannot carry out the salvage pathway because it lacks HGPRT would be 8AzG resistant and would therefore not be killed by the drug. These HGPRT mutants are used in selecting for fused myeloma cells.

The drug aminopterin acts on the major synthetic pathway by interfering with the conversion of tetrahydrofolate to dihydrofolate and preventing a series of one-carbon transfers. Thus in the presence of aminopterin the cell cannot synthesize DNA via the main synthetic pathway, and so must use the salvage pathway. A normal cell can still grow in the presence of aminopterin, but a HGPRT cell cannot because HGPRT mutants cannot carry out the salvage pathway; HGPRT cells are therefore killed in the presence of aminopterin.

When HGPRT myeloma cells are fused with normal cells, the resulting hybridomas are able to grow in the presence of aminopterin because the normal cell contributes functional HGPRT. When hypoxanthine and adenine, which are the precursor molecules used by the enzyme HGPRT in the salvage pathway, are added to the meduim, the hybridoma is able to use the alternate pathway to synthesize DNA. The unfused normal spleen cells die because they are unable to grow for long periods of time in tissue culture, and the unfused myeloma cells are killed by the aminopterin. Thus only the fused hybridomas are able to grow. This process is called HAT selection.

Not all of the hybridomas produced by fusion generate antibody specific to the antigen of interest. Some of the antibodies produced are specific to other

antigens that may have been present in the animal's body at the time the spleen was removed. It is therefore, necessary to clone the hybridomas by diluting the cell suspension and plating the hybridomas in 96 well tissue culture plates at an average of less than one cell per well. Any subsequent growth in that well is assumed to have arisen from one parent cell. Since an antibody-secreting cell produces only one type of antibody, the products of these cloned hybridomas are guaranteed to be of one specificity: they are monoclonal antibodies. These monoclonal antibodies must then be screened for production of antibody against the antigen of interest. Any hybridomas that test positive for desired antibody production are propagated and the antibody periodically harvested. This monoclonal antibody may then be used in analytical methods to test samples for the presence of the particular antigen of interest.

CHAPTER II

REVIEW OF LITERATURE

Researchers have recently developed techniques utilizing monoclonal antibody technology for the detection of a vast array of substances, among them are many of the mycotoxins. Once developed, these tests are not only inexpensive and easily performed, but are among the most sensitive of detection methods available. The following is a compilation of current research on the production of antibodies to various mycotoxins and the further development of monoclonal antibodies to mycotoxins.

The mycotoxins are all low molecular weight compounds which are incapable of inducing an antigenic response because of their small size. To make mycotoxins immunogenic, they must be coupled (as a hapten) to a protein or polypeptide carrier prior to the injection. Bovine serum albumin (BSA) is a commonly used carrier because it is readily available in crystalline form, it has a good immunogenic character and its physicochemical properties are well

known. (Chu et al., 1982). The amount of hapten coupled to each mole of protein carrier is partly responsible for determining the quantity and specificity of the antibodies formed. For this reason an excess of mycotoxin is usually needed to produce a conjugate which will function well as an antigen. (Chu et al., 1982). When sufficient quantities of mycotoxin are difficult to obtain, as is the case with many of the toxins, one of several techniques may be applied to optimize the binding of hapten and carrier and therefore minimize the amount of mycotoxin needed. The technique selected depends on the unique chemical structure of the mycotoxin to be bound and the preference of the researcher. The three most popular procedures are the water soluble carbodiimide technique, the mixed anhydride method, and the reductive alkylation technique all of which are described in detail in the literature (Chu et al., 1982; Gendloff et al., 1986; Sizaret and Malaveille, 1983).

Often the mycotoxin must be converted to a derivative containing a carboxylic acid group or a related functional group before it can be conjugated to a protein polypeptide carrier. Such is the case with the aflatoxins.

1. AFLATOXINS

The aflatoxins are perhaps the most widely studied of the mycotoxins. Their discovery in 1960 as the cause of the "Turkey X" disease and subsequent implication in other mycotoxicoses has served to increase awareness of this and other mycotoxins (Cole and Cox, 1981). There are four acutely toxic, naturally occuring aflatoxins, B1, B2, G1 and G2. The other aflatoxins are derived as metabolic products of animal or microbial systems (M1, M2, P1, Q1) or produced in response to the chemical environment (B2A, G2A, and D2) (Cole and Cox, 1981). The aflatoxins are carcinogenic, mutogenic, and teratogenic. Aflatoxin B1 (AFB1) is one of the most carcinogenic substances known to man. Many antibodies have been formed against the aflatoxins and aflatoxin derivatives by researchers for use in radio-immunoassays (RIA's) or for subsequent development into monoclonal antibodies.

Aflatoxin B1 Antibody

F.S. Chu and I. Ueno (1977) described the production of antibody against AFB1 in 1977. Chu and Ueno found it necessary to convert AFB1, to aflatoxin B1-o-carboxymethyloxime (AFB1-oxime) which has a free carboxyl group suitable for coupling to BSA or

poly-L-lysine (PL). Production of the AFB1-oxime-BSA conjugate was carried out in the presence of a water soluble carbodiimide, 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (EDPC). Following dialysis and filtration the conjugates were used to immunize albino rabbits following the multiple-site intradermal method of Nieschlag et al. (1975). Anti-aflatoxin B1 antibody titer was determined using a binding assay method. The specificity of the antibody for AFB1 against other aflatoxins was determined. Results showed that 10 and 13 moles of AFB1-oxime were successfully conjugated to 1 mole of PL and BSA respectively. Immunizations of rabbits showed that BSA-AFB1-oxime was a good antigen where as PL-AFB1-oxime was not. Maximum antibody production using the BSA-AFB1-oxime conjugate was achieved within 5 to 8 weeks. Sensitivity for AFB1 detection fell in the range of 0.2 to 2.0 ng. While the antibody produced was specific for AFB1, some cross reactivity was noted with aflatoxins B2 and G1 at concentrations 10 times that of B1.

ELISA for Aflatoxin B1

Pestka, Gaur and Chu (1980) continued to work with aflatoxin B1 to develop an enzyme-linked immunosorbent assay (ELISA) which is specific for detecting this

mycotoxin. The researchers made hyperimmune sera by injecting rabbits with aflatoxin B1-carboxymethyloxime-BSA conjugate. This conjugate was then bound to polystyrene microtissue culture plates that had been coated with BSA and glutaraldehyde. A competitve assay was designed whereby a coated plate was exposed to a sample thought to contain aflatoxin B1 to be tested and then to a horseradish peroxidase enzyme - aflatoxin B1 conjugate prepared by Pestka and coworkers (1980). The plate was then treated with an enzyme substrate which reacted colorimetrically when broken down by the peroxidase enzyme. The reaction was terminated by the addition of hydrofluoric acid-edetic acid stopping reagent. Absorbance was detected at 414 nm spectrophotometrically. By treating the plate in this manner a greater absorbance value indicated an increased binding of the peroxidase-aflatoxin B1 conjugate hence, a lesser amount of aflatoxin B1 was present in the sample. When a greater amount of aflatoxin B1 was present, fewer sites were available for the peroxidase-aflatoxin B1 conjugate to bind, less substrate would be converted and the absorbance would be less. The amount of aflatoxin present in a sample was quantitated by comparing absorbance values with controls.

Pestka et al.(1980) recommend this technique for the routine assay of aflatoxin B1 because the sensitivity is equivalent to or better than that of the double antibody RIA (Langone and VanVinakis, 1976), solid phase RIA (Sun and Chu, 1977), HPLC (Stubblefield et al., 1970), and TLC (Horwitz, 1975).

Aflatoxin B1 Monoclonal Antibody

In 1985, Candlish, Stimson and Smith expanded previous aflatoxin research to produce a monoclonal antibody to aflatoxin B1. Candlish and coworkers produced an aflatoxin B1 (oxime)-keyhole limpet haemocyanin conjugate and used it to immunize female Balb-C/NZB F1 hybrid mice. Following immunization the spleen cells were isolated and fused with mouse (x63.Ag8.6.5.3) myeloma cells, The fused cell suspension was distributed into 96 well tissue culture plates and the supernatant liquids screened for specific antibody by indirect non-competitive ELISA twelve days later. Positive hybridomas were then expanded and cloned by a limiting dilution technique. These clones were screened again by ELISA and the best ones were chosen and propogated in tissue culture and as an ascites tumor. Monoclonal antibody harvested from the ascites fluid was conjugated to horseradish peroxidase by a two-step procedure utilizing

glutaraldehyde. This conjugate was used to develop a direct competitive ELISA for the detection of aflatoxin B1. Specificity of the ELISA technique was determined and cross-reactivity was found to be negligible. The sensitivity of the ELISA was 0.2 ng/ml with a working range up to 10 ng/ml for the AFB1. Candlish and coworkers suggested that the monoclonal antibody-based enzyme immunoassay for aflatoxin B1, in addition to its other advantages, is rapid, simple and inexpensive to perform.

In 1981, Haugen et al. published findings which showed that monoclonal antibodies could be formed against AFB1-modified DNA and could be detected by enzyme immunoassay. Monoclonal antibodies were obtained following fusion of mouse P3 x 63 myeloma cells with spleen cells from BALB/c mice that had been immunized with an AFB1-adducted DNA/methylated BSA conjugate. Groopman et al. (1982) demonstrated that these monoclonal antibodies could be used to quantitatively measure AFB1. A modification in DNA of one AFB1 residue in 1,355,000 nucleotides could be detected. In 1984, Groopman et al. expanded this research to develop monoclonal antibodies specific for aflatoxins B1, B2, M1, and the major aflatoxin-DNA adducts for use in a solid-phase immunoassay. The

researchers bound AFB1 to bovine gamma globulin (BGG) by using a modified chemical procedure with m-choloroperoxybenzoic acid. In a typical reaction 40-50 AFB1 residues were bound per molecule of BGG. Ten female BALB/ByCJ mice were immunized with the conjugate and subsequently seven of the ten mice were found to produce significant anti-AFB1 serum titers. The spleen cells from five of these mice were fused with SP-2 myeloma cells and a number of monoclonal antibodies were obtained. Monoclonal antibody 2B11, found to be specific for all the aflatoxins mentioned earlier, was covalently bound to sepharose-4B and was used in a column-based solid-phase immunosorbent assay system (affinity column).

Aflatoxins were added in vitro to phosphate buffer, human urine, human serum and human samples taken from individuals environmentally exposed to the mycotoxins: 90-95% of the aflatoxins were then recovered quantitatively from the samples using the affinity column.

The findings of Groopman and coworkers (1984) indicate that the monoclonal antibody affinity column is useful as a "preparative technique to isolate and purify aflatoxin B1 and its metabolites from complex biologial samples obtained from environmentally exposed

populations." The samples obtained must then be quantitatively analyzed by methods such as HPLC or ELISA.

Groopman and other scientists (1987) recently published data supporting the use of the affinity column for preparing human urine samples for HPLC analysis of AFB1. They have also shown that the measurement of a particular AFB1 DNA adduct is an appropriate dosimeter for estimating exposure status and risk in individuals consuming aflatoxin B1.

"It is well established that AFB1 forms covalent DNA adducts following enzymatic oxidation to a highly reactive 8,9-epoxide which nucleophillically attacks the N7 atom of guanine. This reaction produces the major DNA lesion 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-Gua) and in secondary reactions the other AFB1 DNA adducts". Groopman and coworkers (1987) conducted feeding studies which showed that dietary antioxidants tend to inhibit AFB1 hepatocarcinogenesis when fed simultaneously with the carcinogen. The differential effects of antioxidants on the kinetics of AFB1-DNA adduct formation cause the direct linear extrapolation of AFB1-N7-Gua content in urine to AFB1 dose to be unreliable. The procedure, however, is recommended for estimating exposure to AFB1.

Researchers have produced antibodies against many of the other aflatoxins and aflatoxin derivatives, specifically, aflatoxin B1 dihydrodiol (AFB1-diol) (Pestka and Chu, 1984), aflatoxin B2A (AFB2a) (Gaur et al., 1981), aflatoxin Q1 (AFQ1) (Fan et al., 1984), and aflatoxin M1 (AFM1) (Harder and Chu, 1978). Often the antibodies formed cross-react to recognize the remaining aflatoxins such as aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxicol (Pestka and Chu, 1984).

Aflatoxin B1 Dihydrodiol Antibody

Pestka and Chu (1984) developed antibody against AFB1-diol, a major reactive metabolite of AFB1. They conjugated AFB1-diol to ethylenediamine-modified BSA (EDA-BSA) by a reductive alkylation technique. A 1:25 mole ratio of AFB1-diol to EDA-BSA was achieved by this method and the conjugate was used to immunize New Zealand white rabbits. Within ten weeks high titer AFB1-diol rabbit antibody could be detected by ELISA. Competitive ELISA revealed that the AFB1-diol antibody detected as little as 1 pmol of AFB1-diol per assay.

Aflatoxin B2A Antibody

Gaur and coworkers (1981) produced and characterized AFB2a antiserum. AFB2a was conjugated to

BSA by a reductive alkylation method (the same method later used for AFB1-diol / EDA-BSA conjugation) and the conjugate was used to immunize New Zealand white rabbits by a multiple-site injection method. Anti-AFB2a antibody titer was determined by RIA and ELISA. Cross-reactivity was evident for AFB1, AFB2 and AFM1. The RIA and ELISA sensitivities for AFB1 quantitation were 100 and 10 pg per assay, respectively.

Aflatoxin Q1 Antibody

In 1984, Fan and coworkers published their research on the production and characterization of antibody against AFQ1. Either AFQ1-hemisuccinate or AFQ2a were conjugated to BSA by a mixed anhydride method or reductive alkylation technique respectively and the resulting conjugates were used to immunize albino rabbits. Both RIA and ELISA showed that the antibodies obtained from rabbits following immunization with AFQ2a/BSA conjugate were the most effective in binding AFQ1. Indirect ELISA was capable of detecting as low as 2 ppb of AFQ1 in spiked urine samples and recoveries of 65.8 to 85.8% were obtained.

Aflatoxin M1 Antibody

Harder and Chu (1978) produced and characterized antibody against yet another aflatoxin, AFM1. Aflatoxin M1 was first converted to AFM1-oxime by a method similar to the one described for AFB1 (Chu and Ueno, 1976). The AFM1-oxime was bound to BSA in the presence of EDPC as also described for AFB1 conjugation. Albino rabbits were immunized with the AFM1-oxime/BSA conjugate and after a sufficient time their anti-AFM1 antibody titers were determined by RIA. The antibody produced had greatest binding efficiency for AFM1 and was less efficient for binding AFB1. Weak cross-reactions with aflatoxins Q1, B2a and aflatoxicol were noted. The binding assay for AFM1 was able to detect 1-10 ng/assay.

Aflatoxin M1 Monoclonal Antibody

By building upon the work of Harder and Chu, a monoclonal antibody was produced against AFM1 (Woychik et al. 1984). Once again AFM1 was converted to AFM1-oxime and this was bound to BSA in the presence of EDPC. The AFM1-oxime/BSA conjugate was used to immunize 8-week-old female BALB/c mice. After 8 weeks and 2 booster immunizations the mouse sera was assayed by indirect ELISA for antibody titer. Since anti-AFM1

antibody was detected, the mice were once again boosted and 3 days later their spleen cells were fused with P3-NS1-Ag4-1 myeloma cells. The growing hybridomas were screened by indirect ELISA 10 days postfusion and cells from positive wells were propogated. Cultures that continued to screen as positives after 5 to 8 days of further growth were cloned by a limiting dilution technique. Clones positive for producing anti-AFM1 antibody were grown in culture to produce antibody for further characterization.

2. OCHRATOXIN

Ochratoxin A Antibody

Chu et al. (1976) chose to work with another mycotoxin, ochratoxin A (OA), a potent nephrotoxin produced by a number of Penicillium and Aspergillus species. Ochratoxin A, like the other mycotoxins, is too small to elicit an antigenic response in an animal. It was therefore necessary for the investigators to couple the toxin to a large carrier molecule prior to injecting laboratory animals. Chu and coworkers conjugated OA to a number of different protein carriers by mixing OA with a particular protein in the presence of the water soluble carbodiimide, EDPC. After stirring in the dark for 24 hours, these conjugate

solutions were dialyzed and kept frozen until needed for injections.

A series of immunizations in rabbits over a period of 3 months revealed OA-BSA (bovine serum albumin) to be the best antigen tested. The other conjugates, OA-polylysine 30,000, OA-polylysine 85,000 and OA-lysozyme did not cause as high a titer of antibody to be produced. The antibody formed was found to be specific for ochratoxins A, C and T, but not for ochratoxins B, alpha or other coumarin derivatives by means of a competitive binding assay. The sensitivity of the antibody for OA detection was found to be in the range of 0.5 to 10 ng/0.5 ml sample.

Ochratoxin A Monoclonal Antibody

Ten years after Chu and coworkers produced antibody against OA, Candlish et al. (1986) developed a monoclonal antibody to OA. OA was conjugated to BSA and human gamma-globulin (HGG) in the presence of EDPC by the method of Chu et al. (1976). Female Balb/c x NZB F1 hybrid mice (8 to 12 weeks of age) were immunized with the OA/HGG conjugate. Spleen cells from 2 immunized mice were fused wth X63.Ag8.6.5.3 myeloma cells at a 4:1 ratio and after 12 - 14 days supernatant fluids were screened for anti-OA specific antibody by

indirect ELISA. Positive hybridomas were expanded into flasks, stored in liquid nitrogen as needed and cloned by limiting dilution in the presense of murine spleenocytes as a feeder layer.

One of the cloned hybridomas obtained was found to have a sensitivity of 50 µg/ml of OA in standard solutions. The monoclonal antibody is specific for OA and exhibits a low cross-reactivity for all other naturally occuring metabolites.

Rousseau, et al. (1987) developed another monoclonal antibody against OA by similar procedures and used it in a competitive solid-phase RIA for quantitating OA in porcine kidneys. This technique utilizing the monoclonal antibodies coupled to protein A-Sepharose CL-4B was capable of detecting concentrations of OA as low as 0.2 ng/g in porcine kidneys.

3. RUBRATOXIN B

Rubratoxin B (RB) is a toxic metabolite produced by Penicillium rubrum which has been implicated in the etiology of certain naturally occuring diseases in cattle and swine. RB is hepatotoxic and nephrotoxic and may act synergistically with other toxic

metabolites such as the aflatoxins (Cole and Cox, 1981).

Rubratoxin B Antibody

Davis and Stone (1979) developed an anti-rubratoxin antibody for use in a RIA for RB. RB was coupled to ovalbumin in the presence of EDPC in a 13:1 mole ratio. New Zealand white rabbits were immunized with the conjugate for several weeks. Sera taken from the rabbits was subjected to ammonium sulfate fractionation and the antibody thus precipitated. With the anti-RB antibody and (^{14}C) rubratoxin used in this study the range of the RIA for RB was from 0.1 to 10 µg. Since the structure of RB is dissimilar to the other mycotoxins, any cross-reactivity with other metabolites was not determined. The ability of Davis and Stone to produce anti-RB antibody suggests that the same technique could be expanded to produce monoclonal antibodies against this mycotoxin.

4. ZEARALENONE

Zearalenone is a naturally occuring metabolite produced by members of the genus Fusarium. Though they exhibit a low order of toxicity, zearalenone and its derivatives zearalenol and zeranol exert extensive estrogenic effects on the mammalian reproductive system. Zearalenone has been detected as a contaminant in wheat, corn, barley and bovine milk: therefore the need exists for screening of commodities slated for human or animal consumption. Many researchers have been working to produce antibodies to zearalenone and its analogues for widespread use in the rapid screening of agricultural products.

Zearalenone Antibody

S.N. Dixon first published a procedure for the production of antibody against zeranol in 1980. Zeranol was connected to zeranol hemisuccinate and bound to BSA. Sheep were immunized with the conjugate and their sera tested for specific antibody beginning 3 weeks after the initial immunization. "The sensitivity of the assay procedure allows the detection of zeranol and/or its main metabolite zearalanone between 100 and 1000 pg."

Pestka et al. (1985) published a procedure for immunizing swine to produce antibody against zearalenone. The production of antisera in swine was investigated for potential use as a prophylaxis against zearalenone hyperestrogenism. Male pigs (4 - 5 weeks old) were immunized with zearalenone-6'-carboxymethyloxine which had been coupled to BSA by a mixed anhydride procedure. Four different immunization protocols were attempted and the one eliciting the highest antibody response as determined by ELISA was selected. The anti-zearalenone antibody produced by this procedure was characterized and found to be specific for zearalenone with relatively low cross-reactivity for analogues of the mycotoxin. Indirect competitive ELISA for zearalenone using this antiserum had an assay detection limit of 10 ng/ml for the toxin. Pestka et al. (1985) speculate that the immunization protocol developed might be applicable to testing active immunization as a method for preventing zearalenone mycotoxicosis in swine. "In addition, antisera collected from immunized swine might be utilized in passive therapy for neutralizing the estrogenic effects in swine already exposed to zearalenone."

Thouvenot and Morfin (1982) demonstrated once again that it is possible to raise antibody against zearalenone. The zearalenone mycotoxin and its derivative, zearalanone, were bound to BSA in the presence of a water-soluble carbodiimide. Three-month-old, 60 kg male pigs were immunized with the conjugates and after a sufficient time their sera were tested for the presence of specific antibody by RIA. The porcine antibodies detected were found to be specific for the resorcyclic acid lactones of structural resemblance to zearalenone. "This specificity made the antibodies useful for a RIA of zearalenone and zearalanol which may be found in human and animal sera. The range of the assay was between 0.25 and 10 ng. The limit of detection was 5 ppb (5 ng/ml) in human serum."

Zearalenone Monoclonal Antibody

Two groups of researchers have developed monoclonal antibodies against zearalenone derivatives. Dixon et al. (1987) produced these monoclonal antibodies for anticipated use in the rapid screening of agricultural commodities. Carter et al. (1984) developed anti-zearanol antibodies in order to monitor the residues of the agent in urine, bile, feces and

tissue extracts of animals treated with zearalenone to improve their average daily liveweight gain.

Dixon et al. (1987) conjugated zearalenone to BSA for use as an immunogen in BALB/c mice. Hyperimmune speen cells from the immunized mice were fused with NS-1 myeloma cells and the resulting hybridomas were screened by ELISA for production of specific antibody. Those hybridomas found to secrete anti-zearalenone antibody were cloned by a limiting dilution technique and rescreened by ELISA. Positive clones were selected and propagated as an ascites tumor in histocompatible mice. A competitive indirect ELISA employing this antibody was capable of detecting zearalenone at 0.5 ng/ml.

Carter et al. (1984) produced monoclonal antibodies using two haptens, zearanol-7-hemisuccinate coupled to BSA and zeranol-16-carboxypropyl ether coupled to human serum albumin. The researchers were successful in developing four independent cell lines secreting monoclonal antibodies to zeranol. Some cross-reactivity was noted but only against other zearalenol derivatives.

5. TRICOTHECENE TOXINS

The tricothecenes are a group of mycotoxins produced by various species of fungi imperfecti. They show a wide range of biological activity and have been strongly implicated in natural intoxications of animals and man. "These mycotoxicoses are moldy corn toxicoses of cattle, swine, and poultry in the United States; Akakabi-Byo disease in Japan; Stachybotryo-toxicosis in the U.S.S.R.; alimentary toxic aleukia; and dendrochiotoxicosis. Thus, they are potentially one of the most important mycotoxin groups to animals and man" (Cole and Cox, 1981).

T-2 Antibody

Over the past decade researchers have been successful in producing antibodies against many of the tricothecene toxins. Chu and coworkers developed antibody against T-2 toxin in 1979. Rabbits were immunized with a BSA-T-2 hemisuccinate conjugate over a period of several weeks. The conjugate was prepared by the same procedure described earlier for aflatoxin conjugation (Chu and Ueno, 1977). The sera was analyzed by RIA for presence of antibody specific for T-2 toxin. The sensitivity of the binding assay for the detection of T-2 toxin was in the range of 1 to 20

ng per assay. The antibody had greatest binding efficiency for T-2 toxin and lesser cross-reactivity with other tricothecenes tested.

Deoxyverrucarol Antibody

In 1984, Chu and coworkers produced antibody against another tricothecene toxin, deoxyverrucarol (DOVE). BSA was coupled to DOVE-hemisuccinate by the water soluble carbodiimide technique described earlier for aflatoxin (Chu and Ueno, 1977). The DOVE/BSA conjugate was used to immunize female albino rabbits by a multiple-site intradermal injection technique. At the appropriate time the rabbits' sera was assayed for presence of DOVE specific antibody by RIA. The antibodies detected were highly specific for DOVE only and not for the common tricothecene backbone.

Diacetoxyscirpenol Antibody

Chu and coworkers also described the production of antibody to diacetoxyscirpenol (DAS) in 1984. Hemiglutarate derivatives of DAS coupled to BSA were found to be immunogenic in rabbits. Antibodies against DAS were obtained and competitive RIA revealed that the antisera showed high specificity and practically no cross-reactivity. Chu and coworkers recommended that with a method now available for producing antibodies,

alternate immunoassays for DAS, such as ELISA, should be developed.

In 1985 Zhang and coworkers presented an improved method for the production of antibodies against T-2 toxin and DAS in rabbits. This method differed from previous procedures in that immunogens were prepared by conjugating carboymethyl oxime derivatives of the toxins to BSA. Antibodies against both toxins were detected as early as 4 weeks after immunization. This method of antibody production was found to be superior because of the increased stability of the immunogens.

Other Tricothecene Toxin Antibodies

Chu and his associates were instrumental in the production of antibody to two other tricothecene toxins, 3′-OH-T-2 toxin (Wei et al. 1986) and deoxynivalenol triacetate (Zhang et al. 1986). Antibodies to both of these toxins were formed by immunizing rabbits with hemisuccinate derivatives of the toxins coupled to BSA. The detection limit for both toxins by RIA was about 0.1 ng per assay.

A generic antibody was produced against all group A tricothecenes by Wei and Chu (1987) for possible use in the analysis of tricothecenes or as a prophylactic agent for neutralizing the toxicities of type A

tricothecene mycotoxins. The antibody was produced after immunization of rabbits with a conjugate of T-2 toxin and BSA.

T-2 Toxin Monoclonal Antibodies

Hunter et al. (1985) prepared mouse monoclonal antibodies against T-2 toxin because of previous problems of standardization and low titers of polyclonal antisera produced. Female BALB/c mice were immunized with T-2 toxin conjugated to goat immunoglobulin G (GIgG) by the method of Chu et al (1979). Spleenocytes were harvested 4 days post-immunization and fused to murine myeloma cells. Hybridomas were screened by ELISA and those found to be positive were cloned by limiting dilution and adapted as ascites tumors in mice. Two monoclonal antibodies were found to be specific for T-2 toxin. The specificity of the ELISA with both monoclonal antibodies was somewhat better than that experienced with polyclonal antisera. The ELISA utilizing the monoclonal antibody could detect 50 ng of T-2 toxin per assay.

Gendloff et al. (1987) also produced monoclonal antibody against T-2 Toxin. The toxin was converted to T-2 hemisuccinate and coupled to BSA by the method of

Chu et al. (1979). Monoclonal antibodies were obtained by the usual methods and screened by ELISA. A T-2 toxin specific monoclonal antibody was derived and used in a competitive indirect ELISA for detection of the mycotoxin. Sensitivity of the assay was found to be 10 ng/ml which is more sensitive than the previously reported T-2 monoclonal antibody system described by Hunter et al. (1985).

Application of T-2 Toxin Antibody

An interesting application of a monoclonal antibody produced against T-2 toxin was reported by Feuerstein et al. (1985). It has been suggested that T-2 toxin was the major component of "yellow rain" which may have been used as a chemical warfare agent in Southeast Asia. T-2 toxin is an inhibitor of protein synthesis and is suspected as a cause of the highly fatal disorder, alimentary toxic aleukia, in humans. No specific therapy has been established for the disease. "Previous studies have indicated that antibodies can neutralize in vitro toxic effects and provide in vivo protection against drugs and certain low molecular weight toxins" (Feuerstein et al. 1985). These researchers showed that monoclonal anti-T-2 antibody can neutralize the in vitro protein synthesis inhibition of T-2 toxin and can protect rats against

lethal T-2 toxemia. A dose of the anti-T-2 monoclonal antibody given 60 minutes after the administration of a lethal dose of T-2 toxin resulted in an increased time to death and a 45% survival rate. These data were the first demonstration of effective prophylaxis and therapy for T-2 toxemia.

CHAPTER III

MATERIALS AND METHODS

1. PRODUCTION OF BSA/CITRININ CONJUGATE

Citrinin Source and Conjugation

Pure crystalline citrinin was purchased from Sigma Chemical Company (St. Louis, MO). Known amounts of citrinin and bovine serum albumin (BSA, Sigma) were mixed together, incubated at 37°C for two hours and then dialyzed in dialysis tubing having a molecular weight cutoff of 2000 (Spectrum Medical Industries Incorporated, Los Angeles, CA) against a barbital/sodium barbital buffer (pH 8.6) (Damodaran, 1977). The dialysis buffer was analyzed for citrinin concentration using a Waters Associates HPLC and a variation of the HPLC method of Phillips et al. (1980). A model 6000A solvent delivery system and a U6k septumless injector were utilized in conjuction with a u Bondapak C18 reverse phase column, a model 420C and 420E fluorescence detector (excitation wavelength = 360 nm, emission wavelength = 500 nm) and a model 440 absorbance detector (absorbance wavelength = 340 nm). The eluting solvent consisted of 55 parts phosphoric acid (0.25 N) to 35 parts acetonitrile to 10 parts

2-propanol (all purchased from Sigma). The eluting solvent was filtered through a 0.2 um membrane filter (Gelman Instrument Co.) and was degassed by stirring under vacuum. Dialysis controls contained citrinin, but no BSA in an equal volume of buffer. Comparison of the two dialysis buffer solutions provided data for the determination of the molar binding ratio. Samples of the conjugate were dialyzed for one or seven days to determine stability (Chu et al., 1976). Conjugates (BSA + citrinin) were removed from the dialysis tubing, lyophilized and stored until needed.

Immunogen Preparation

The citrinin/BSA conjugate production procedure was scaled up slightly to produce quantities sufficient for immunization purposes. The expanded procedure involved the conjugtion of 700 µg of citrinin with 1.85 mg of BSA by the method described earlier. The conjugate solution was exhaustively dialyzed against 3.5 l of barbital/sodium barbitol buffer. Following dialysis, the conjugate was lyophilized overnight and its weight calculated from the known weights of the starting materials. Dried conjugate was stored at -20°C until needed.

2. IMMUNIZATION PROTOCOL

Lyophilized citrinin/BSA conjugate was resuspended in distilled water to a concentration of approximately 100 µg/ml. Male RBF mice (six-to-eight weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and initially immunized with 0.5 ml (40-50 µg) of conjugate emulsified with an equal volume of Freund's complete adjuvant (Sigma). Second and third immunizations at four-to-five week intervals consisted of 0.5 ml (40-50 ug) of conjugate in a 1:1 ratio with Freund's imcomplete adjuvant (Sigma). One week following the third immunization, serum was collected from the retrobulbar plexus of each mouse and antibody titer was determined by indirect ELISA. At least one week after the third injection and four-to-nine days prior to fusion, the mice were given a fourth immunization of 40-50 µg of conjugate with no adjuvant. All immunizations were given subcutaneously at three to four sites along the back. Mice used in this study were housed in the University of Tennessee Veterinary Teaching Hospital Animal Facilities.

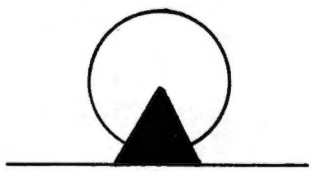
3. INDIRECT ELISA PROCEDURE

The indirect ELISA procedure used in this study is outlined in figure 3. Tissue culture plates (96-well;

1. Immobilized antigen (citrinin)

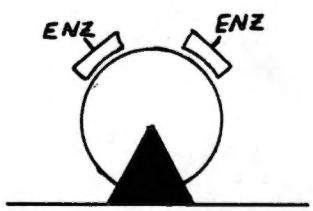
Wash

2. Addition of mouse serum containing specific antibody (anti-citrinin)



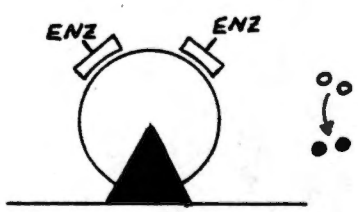
Wash

3. Addition of enzyme-second antibody conjugate (anti-mouse Ig)



Wash

4. Addition and conversion of substrate



Source: Sigma Catalogue. 1987. Sigma Chemical Company, St. Louis, MO.

Figure 3. Indirect ELISA: detection of specific antibody.

American Scientific, Atlanta, GA) were coated with antigen by incubation of 50 µl of citrinin/sperm whale myoglobin (SWM) conjugate (prepared in the same manner as the citrinin/BSA conjugate described earlier) per well at 4°C overnight. Unbound conjugate was removed from the plates with three washes of phosphate buffered saline (PBS, pH 7.4) (Appendix A) containing 0.20% Tween 20 (PBS-Tween). Blockage of free protein binding sites was achieved by incubation of 50 µl per well of PBS Tween containing 0.3% goat serum (Sigma) for 60 minutes at 37°C. The plates were then washed three more times in PBS-Tween and triplicate aliquots of antiserum, serially diluted in PBS-Tween, were added to the wells and incubated for 60 minutes at 37°C. Triplicate wells of normal (preimmune) mouse sera served as a control. Unbound antibody was removed by washing three times in PBS-Tween, and 50 µl of goat anti-mouse IgG horseradish perioxidase conjugate (Sigma) diluted 1:2000 in PBS-Tween containing 3% goat serum was added to each well. The plates were then incubated for 60 minutes at 37°C and washed six times in PBS-Tween. Bound antibody enzyme conjugate was measured by addition of an enzyme substrate solution containing 1 mg/ml of orto-phenylenediamine (OPD) and 1 µl/ml of hydrogen peroxide in citrate buffer (pH 6.0). Following development of the substrate for 15-30

minutes at room temperature the reaction was stopped by the addition of 25 µl of 0.25 M sulfuric acid to each well. Absorbance was read at 405 nm on a model EL 310 EIA Autoreader (Bio-Tek Instruments, Inc., Burlington, VT).

4. HYBRIDOMA PREPARATION

Cell Line and Cell Culture Media

The myeloma cell line used for fusion, FOX-NY, was provided by B.T. Rouse, Department of Microbiology, University of Tennessee. The myeloma cells were thawed from a frozed state and maintained in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS) (Sigma). Hybridomas were cultured in hybridoma complete medium (HCM) (Appendix A). Fusions were carried out in Dulbecco modified Eagle medium (DMEM) (Gibco) containing 15 mm Hepes buffer (American Scientific).

Cell Fusion Procedure

A total of four-to-seven days after the final injection of citrinin/BSA conjugate, two mice were sacrificed and saturated in 70% ethanol. Their spleens were aseptically removed and placed into serum-free medium, Hank's Balanced Salts Solution (HBSS, Gibco).

Spleens were dissociated into single cell suspensions by forcing them through a stainless steel strainer and repeatedly pipetting them. The cells were then pelleted by centrifugation at 200xg for ten minutes, resuspended in tris-ammonium chloride (Appendix A) and incubated at 37°C for five minutes to lyse the red blood cells. Following incubation HBSS was added to dilute away the tris-ammonium chloride and the cells were again pelleted by centrifugation. The spleen cells and myeloma cells were then each washed three times separately in HBSS and resuspended. Both types of cells were counted and combined in a 50 ml conical polypropylene tube in a ratio of ten spleen cells to one myeloma cell. Following centrifugation, the supernatant was decanted and 1 ml of fusion mixture (Appendix A) containing polyethylene glycol (PEG, Sigma) was added drop by drop over 60 seconds with agitation. Immediately following centrifugation, the fusion mixture was slowly diluted away with HBSS, and allowed to stand at room temperature for five minutes before it was once again pelleted by centrifugation. the supernatant was discarded and the cells were suspended in HCM using a pipet to gently bubble the mixture. The cells were allowed to stand at room temperature for 30 minutes, then they were gently mixed and their concentration adjusted to 0.5×10^6 immune

cells per ml. The cells were distributed (50 µl/well) into 96-well tissue culture plates and allowed to incubate for 24 hours at 37°C.

The next day, 50 µl of a 2x HAT (hypoxanthine, aminopterin, thymidine) solution (Sigma) was added to each well. After one-to-two weeks of growth in HCM + HAT media the HAT was withdrawn and the selected hybridomas were cultured in HCM + HT (Sigma) (no aminopterin) for several weeks. The remaining hybridomas were then cultured in HCM only.

Screening of Hybridomas

The hybridomas were expanded into 24-well plates before their supernatants could be assayed by ELISA for the presence of anti-citrinin antibody. The ELISA technique described earlier was also used to screen the hybridomas.

5. CLONING PROCEDURE

Preparation of Spleenocyte Feeder Layer

Hybridomas found to be secreting antibody against citrinin were cloned by a limiting dilution technique. Prior to cloning the hybridomas, five BALB/c mice were sacrificed and their spleens aseptically removed and prepared for use as a feeder cell layer. A layer of

spleenocytes is necessary for the subsequent growth of the hybridoma clones. The five spleens were minced through a fine wire mesh screen made of a stainless steel and suspended in 15 ml of HBSS in a 50 ml polypropylene centrifuge tube. The cells were pelleted by centrifugation at 1000 rpm for six minutes. The pellet was resuspended in 10 ml tris-ammonium chloride and allowed to incubate at 37°C for three minutes. Following incubation the tube was filled with HBSS and the cells once again pelleted by centrifugation. The cells were washed again in HBSS, pelleted and resuspended in 40 ml of RPMI 1640 containing 10% FBS. The cells were then irradiated with a dose of approximately 2400 rads of radiation to prevent the spleenocytes from reproducing or secreting antibody. Following irradiation the spleenocytes were again pelleted by centrifugation and resuspended in HCM plus HT media. The cells were counted and adjusted to 5 x 10^6 cells/ml and dispensed into 96-well tissue culture plates. Plates were incubated at 37°C in a five percent CO_2 atmosphere overnight.

Cloning Procedure

After a 24-hour incubation, the spleenocyte feeder layer was examined for presence of contamination and finding none, cloning was begun. The cell number of

the hybridoma to be cloned was calculated using a hemocytometer and the appropriate amount of HCM media added to effect a 50 cell/ml concentration. This solution was dispensed (100 µl/well) over one third of a 96-well plate containing the feeder cell layer. This resulted in a final concentration of approximately five hybridoma cells being dispensed in each well. A second solution of hybridoma cells was prepared at a concentration of 10 cells/ml by diluting the original solution one-to-five and 100 µl of this suspension was dispenced into each well of the middle third of the 96-well plate (resulting in one hybridoma cell/well). This second solution was diluted again one-to-five and the resulting two cell/ml solution was dispensed 100 µl/well into the remainder of the wells in the 96-well plate. This portion of the plate then contained statistically 0.2 cells/well. Following one-to-two weeks growth, the plates were examined and if less than one third of the wells containing one cell/well exhibited growth then the cloning dilutions were made correctly and clones could be selected from the one cell/well or 0.2 cell/well sections of the plate because these cell cultures arose from one parent cell. The clones were all propagated separately and expanded to large (75 cm^2 surface area) tissue culture flasks and their supernatant fluids screened again by ELISA.

Cell Freezing Procedure

Any clones or parent hybridoma cell lines that were found to be positive for production of anti-citrinin antibody were frozen and stored in liquid nitrogen for future characterization and study of antibody. Cell lines to be frozen were grown to a concentration of 10^6 cells/ml and 10 ml of this solution was pelleted by centrifugation at 1000 rpm for six minutes. The pellet of cells was resuspended in 1 ml of FBS and transferred to a 2 ml cryogenic tube. To this tube was added 1 ml of a sterile 20% dimethyl sulfoxinde (DMSO) (Sigma) - FBS solution. The cryogenic tube was sealed, placed in a model R204 cell freezer (Planer Products, Ltd., Sunbury-on-Thames, England) and frozen at a rate of -1°C/second to a temperature of -80°C before being transfered to a liquid nitrogen storage tank.

CHAPTER IV

RESULTS AND DISCUSSION

1. DETERMINATION OF BSA/CITRININ MOLE BINDING RATIO

Two replicates of an experiment were conducted to determine how many moles of citrinin were bound per mole of BSA by the conjugation technique described earlier. The first replicate utilized both HPLC fluorescence and spectrophotometric data and all chromatographic peak areas were calculated by hand. This experiment showed that an approximate 19:1 mole ratio of citrinin to BSA may be achieved in a conjugate allowed to dialyze for seven days prior to analysis (Appendix B).

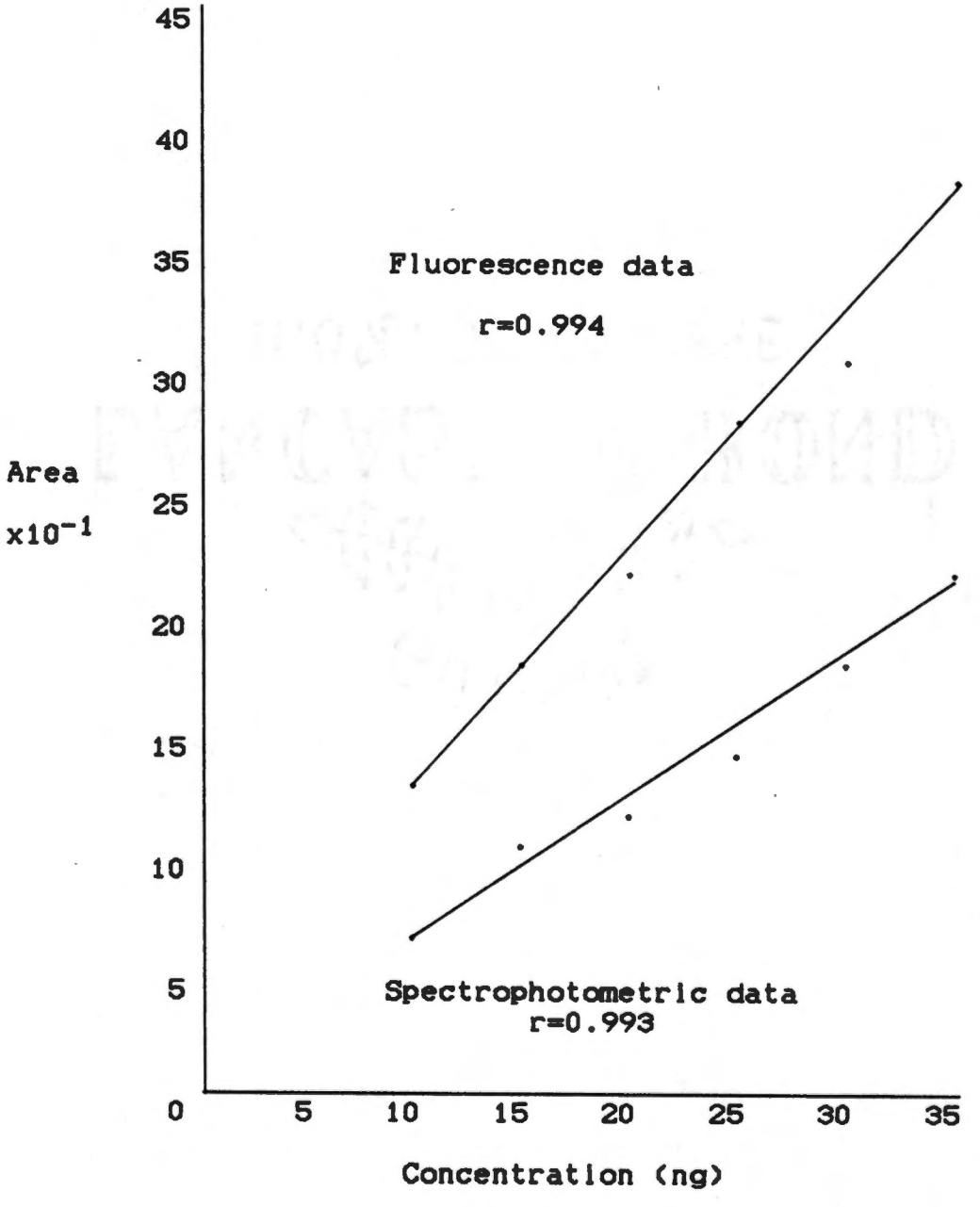
Previous work by Damodaran (1975) suggested that citrinin would bind to human serum albumin but no attempt was made to elicit the binding ratio. To determine the 19:1 binding ratio of citrinin to BSA many calculations were made. The retention time of citrinin in this system was approximately five minutes and some peak tailing was evident. For this reason all chromatograms were measured by triangulation and area

was computed from the height and the width at the base of each peak according to the formulas: $W_b = 1.7 \times W_{.5H}$ and Area = $H \times W_{.5H}$. The averages of the peak areas for the standards as detected by fluorescence versus UV are summarized in Table 1. The equation for the fluorescence standard curve and the spectrophotometric standard curve was calculated by linear regression. Both curves were linear over the range from 10 to 35 ng (Figure 4). As expected, the slope of the fluorescence standard curve was greater than that of the spectrophotometric standard curve thus showing that the fluorometric detection is the most sensitive of the two techniques. For this reason, the fluorometric analysis was used in subsequent calculation of the binding ratio. The samples which were analyzed showed a smaller standard deviation between replicates when fluorescence data was used than when UV data was inspected. This supports the hypothesis that the fluorescence analysis was more precise than UV. The percent recovery for this method was calculated to be 101% as analyzed in the control citrinin buffer.

Actual mole binding ratios were calculated using the fluorescence data. The average peak area for the citrinin control dialysis buffer was substituted into

Table 1. Analysis of citrinin from barbital/sodium barbital dialysis buffers with and without BSA.

Sample	Fluorescence Area (mm^2)	UV Area (mm^2)
Citrinin/BSA		
Average	190.7	115.8
Standard deviation	12.1	14.2
Citrinin control		
Average	227.1	129.3
Standard deviation	12.9	17.9



Fluorescence equation Y = 9.7(x) + 31.9
Spectrophotometric equation Y = 5.7(x) + 13.6

Figure 4. Citrinin standard curves (HPLC).

the equation for the fluorescence standard curve, the concentration of citrinin was found to be 20.11 ng. Similarly, the concentration of citrinin in the dialysis buffer from the citrinin/BSA conjugate yielded a concentration of 16.36 ng (Figure 5). The difference between these two concentrations is 3.75 ng. If this difference is divided by the amount of citrinin found to be present in the control buffer and the result multiplied by 100, it was found that this 3.75 ng difference represents an 18.65% difference in the amount of citrinin found in the dialysis buffer of the control solution over the amount of citrinin found in the solution containing BSA. Since prior to dialysis the concentrations of citrinin within both of the dialysis tubes was the same, it can be assumed that the 18.65% of citrinin missing in the dialysis buffer of the BSA containing solution has, in fact, been bound by the BSA and is, therefore, not free to pass through the dialysis membrane. This leads to the conclusion that if 18.65% of the original 100 µg of citrinin that was incubated with the 1 ml of 0.004 mM BSA (264 µg BSA) was indeed bound, then 18.65 µg of citrinin is bound per 264 µg of BSA. Simple conversions show a mole ratio of 18.65 moles of citrinin bound by each mole of BSA as the calculations in Appendix B indicate.

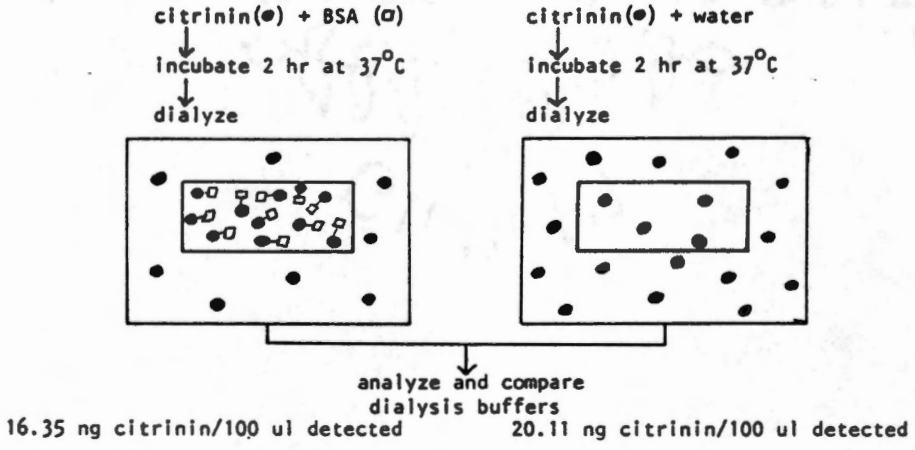


Figure 5. Conjugate production and determination of mole binding ratio.

The second replicate of this experiment examined the buffer solutions used to dialyze conjugate for one day only. Only fluorescence data was utilized because, for reasons stated earlier, it is considered more precise (Table 2). Peak areas were calculated by a computerized integrator. Calculations identical to those made in the first replicate were used to determine a mole binding ratio of approximately 31 moles of citrinin per mole of BSA.

The reverse phase HPLC technique for analysis of citrinin concentration demonstrated that the mere mixing and incubation of proper proportions of citrinin and BSA was sufficient to allow conjugation to proceed. The binding studies undertaken show that a 31:1 mole binding ratio of citrinin to BSA was present after only one day of dialysis, however, if the dialysis was allowed to proceed for one week, the mole binding ratio was apparently reduced to 19:1. These data may demonstrate some instability in the conjugate. The lower 19:1 mole ratio may result from the BSA releasing previously bound citrinin during the week-long dialysis. The higher 31:1 ratio, however, may be due to incomplete dialysis, assuming the unbound citrinin did not reach equilibrium overnight. It is also possibile that the differences are attributable to

Table 2. Molar binding ratios for citrinin and BSA in barbital/sodium barbital buffer dialyzed for one and seven days.

Length of dialysis	mole binding ratio (citrinin:BSA)
7 days	19:1
1 day	31:1

variability in other factors between the two replicates. When injected into a laboratory animal, an antigen with a hapten/carrier mole ratio of less than 10:1 is associated with the production of low affinity antibodies obtained only in low titers. However, when the mole ratio is 20:1 or better it is possible to induce the synthesis of antibodies with high titers (Worsaae, 1978). Since the citrinin/BSA conjugate had a mole ratio near 20:1 the decision was made to use this conjugate as an immunogen in the production of anti-citrinin antibodies and relatively high titers of antibodies with a high affinity for citrinin were anticipated.

2. DEVELOPMENT OF ELISA FOR DETECTION OF ANTI-CITRININ ANTIBODY

Binding of Citrinin to ELISA Plate

Several studies were conducted to determine a suitable method for coupling citrinin to an ELISA plate for subsequent reaction with antibody. In one experiment crystalline citrinin was dissolved in ethanol and diluted across an ELISA plate. The ethanol on the plate was not allowed to evaporate before the plate was washed. Evidently the ethanol had such a strong affinity for the citrinin it did not allow the

citrinin to bind to the plate. Other experiments were conducted using water to dissolve the citrinin before dispensing it on the plate. Bicarbonate buffer was also tried. Neither solvent was found to be suitable for binding citrinin to the ELISA plate. Finally, a SWM/citrinin conjugate was made and diluted in bicarbonate buffer (pH 9.6) and placed in the wells of an ELISA plate. This procedure successfully bound citrinin to the ELISA plate and a 1:16 dilution of the SWM/citrinin conjugate was found to produce the best results.

Analysis of Polyclonal Sera

Analysis of the pooled sera from three of the immunized mice by ELISA showed that the immunizations of BSA/citrinin conjugate were successful in eliciting the production of anti-citrinin antibody. Dilutions of the immune sera 1:50 and 1:100 gave absorbance values well above those of the normal control sera (Appendix B). The immunizing conjugate of BSA/citrinin was also used as an antigen in this ELISA. As expected many more antibodies were detected against BSA/citrinin than SWM/citrinin. The assay of the immune sera was, nevertheless, encouraging and the decision was made to attempt to create a hybridoma.

Analysis of the pooled sera from three of the immunized mice offered definitive proof that anti-citrinin antibodies had been produced in response to the BSA/citrinin immunogen (Table 3). The use of the BSA/citrinin conjugate as an antigen on the ELISA plate revealed a large number of antibodies present. Most of these antibodies were against BSA as expected, since BSA was used as the carrier molecule in the immunogen. The absorbance values for the SWM/citrinin conjugate, while not as large as the BSA/citrinin values, were of greater importance because they represented the actual anti-citrinin antibodies. The mice had never been exposed to SWM and thus produced no antibodies against it. Differences in absorbance values detected between the control sera (0.12) and the immune sera (0.22) where SWM/citrinin served as the ELISA antigen were directly attributable to the presence of citrinin specific antibodies. These results were sufficient to warrant the further development of a hybridoma cell line secreting these antibodies.

Table 3. ELISA of sera from immunized (citrinin/BSA) and control mice.

Antibody source	ELISA antigen SWM/citrinin	ELISA antigen BSA/citrinin
Sera-immunized mice	0.223[a]	1.331
Sera-control mice	0.120	0.103

[a]Absorbance values at 405nm

3. HYBRIDOMA PRODUCTION AND ASSAY

First Fusion

Three separate fusions of spleen cells from the immunized mice with FOX-NY myeloma cells were attempted. The first fusion involved three mice and was carried out nine days after the mice were administered their third dose of immunogen. The fusion protocol was followed implicitly and approximately two weeks later 27 actively growing hybridomas were selected. As these hybridomas reached stationary growth phase their supernatants were screened by indirect ELISA for presence of anti-citrinin antibody. All of these hybridomas tested negative, nevertheless, they were expanded into large tissue culture flasks and frozen for future study. Prior to freezing, 11 of the most active hybridomas were selected and approximately five ml of supernatant fluid was taken from each. These samples were concentrated by two-to-three times by filling sections of dialysis tubing with the supernatant and placing the tubing in a bed of lypholization crystals (Gelman Sciences, Inc., Ann Arbor, MI). Concentration was also attempted through the use of disposable concentrators (Amicon, Danvers, MA). These disposable concentrators were filled with two ml of supernatant and subjected to centrifugation

for 15 minutes at 1000 rpm. Subsequent analysis of the concentrated supernatant solutions by indirect ELISA was also negative for presence of anti-citrinin antibody.

The length of time between the final immunization of the mice and fusion of their spleen cells with myeloma cells can be a critical factor in the development of a productive hybridoma. Many researchers recommend a three to four day interval between the final booster injection and fusion (Rousseau et al., 1987; Candlish et al., 1986). In the first fusion attempt nine days elapsed before fusion was carried out. This may have had some effect on the hybridomas that were formed. The maximum number of spleen cells secreting antibody are present three-to-four days post-fusion. A nine day lapse may have allowed the anti-citrinin immune response to climax and recede prior to fusion. This may explain why none of the hybridomas produced in the first fusion were found to generate anti-citrinin antibody.

It is also worthy to note that none of the antibody concentration techniques were able to yield a positive antibody response. Neither technique reduced the volume of the supernatant fluid by much more than 50%.

Other possible reasons for the failure of the first fusion to produce a hybridoma generating citrinin specific antibody are several. The immunogen may not have been a stable enough conjugate, although this is unlikely because positive polyclonal antibody was detected prior to fusion. Most immunogens have several antigenic sites and as a result induce the expansion of several clones of antigen-reactive B-cells (one clone for each determinant). The response of the mouse to the whole antigen is polyclonal: the response to each antigenic determinant is monoclonal (Golub, 1987). It is statistically quite possible, especially given the relatively long period of time between the final immunization and fusion, that the procedure did not succeed in fusing the particular B-cell secreting antibody to the most specific antigenic determinant on citrinin. It is also possible that all of the parameters involved in the indirect ELISA procedure used to assay the hybridomas were not optimized.

Second Fusion

The second fusion attempt was unique in that it utilized the spleenocytes from just one mouse that had been exposed to the BSA/citrinin antigen on three separate occasions. The mouse was sacrificed, its spleen removed, dissociated into a single cell

suspension and placed in a tissue culture plate. The spleenocytes were then stimulated in vitro by the addition of 30 µg of the BSA/citrinin conjugate. The following day the spleenocytes were fused to myeloma cells by the normal procedure. Within two weeks, eight actively growing hybridomas were selected and expanded in culture. Subsequent analysis by indirect ELISA revealed no anti-citrinin antibodies present in the supernatant fluids.

The second fusion attempt was unsuccessful in producing a hybridoma generating citrinin specific antibody. The in vitro sensitization technique attempted produced no better results than the normal in vivo immunization procedure. The eight hybridomas produced by fusion all grew well and appeared in the classical clumping formation characteristic of hybridomas, yet analysis revealed no citrinin specific antibodies. The reasons for failure of the second fusion could include any of those described for the first fusion in addition to the prospect that in vitro sensitization was unsatisfactory.

Third Fusion

The third and final fusion required the spleens of two mice immunized three times: the last immunizaton

was performed four days prior to fusion. Approximately two weeks after fusion 19 hybridomas were selected and expanded in culture. These hybridomas were screened by indirect ELISA and culture number 2B.2 was found to be slightly positive for production of anti-citrinin antibodies. Since the mice used to generate the antibodies were immunized with BSA/citrinin, in which the hapten was conjugated to an amino acid residue in BSA, this hapten-carrier system may generate antibodies that recognize certain epitopes that BSA and SWM may have in common. Therefore, to eliminate the possibility of false positive results, the supernatant fluid from hybridomas 2B.2 was assayed for activity against the immunogen conjugate, BSA/citrinin; the ELISA conjugate, SWM/citrinin; and against solutions of pure BSA and SWM (Table 4). Indirect ELISA of hybridoma 2B.2 shows that, indeed, some SWM epitopes were recognized by the antibodies produced by the mice, but the absorbance readings are slightly higher for the SWM/citrinin conjugate. This suggests that some citrinin specific antibodies are present in a low titer. The positive control in this experiment (polyclonal sera taken from the immunized mice) shows a much higher titer of citrinin specific antibody and no indication of cross reactivity with SWM.

Table 4. Assay of hybridoma 2B.2 by ELISA.

Sample	BSA	SWM	BSA/clt	SWM/clt
2B.2	0.032	0.125	0.069	0.136
+ control	0.702	0.131	0.768	0.254
- control	0.022	0.037	0.019	0.041

Absorbance values at 405 nm.
+ control = polyclonal sera from immunized mice.
- control = sera from non-immunized mice.

The third fusion attempt was successful. The one positive hybridoma detected, 2B.2, was found to generate citrinin specific antibody. This successful fusion was performed at the peak of the immune response, four days after the last immunization. This tends to support the idea that successful hybridomas formation is dependent upon the proper timing of the immunization and fusion schedules.

4. CLONING OF HYBRIDOMAS

Cloning and Initial Assay

Hybridoma 2B.2 was cloned by the usual procedure and within two weeks 31 clones were selected and expanded into 24-well plates for analysis. Indirect ELISA of the supernatant fluids from the 31 clones was carried out against the four antigens described earlier. Triplicate wells were assayed for response of the antibodies to each antigen. Averages and standard deviations were calculated for the triplicate well readings and statistical significance was assigned to the clone if the average minus two times the standard deviation of the SWM/citrinin readings was still greater than the average plus two times the standard deviation of the SWM values. No overlap within a two standard deviation range represents a probability level

of 0.05. Only the antibody generated by clone number 2B.2.2.19 was found to be statistically significant by this procedure. A malfunction of the ELISA reader made a complete analysis of all the clones impossible.

Final Analysis

Since primary analysis of the clones was incomplete, the 31 clonal cell lines were expanded into small tissue culture flasks and again assayed by indirect ELISA. Statistical significance was assigned as before and three clonal cell lines, 2B.2.2.13, 2B.2.2.18 and 2B.2.2.19, were found to be significant at the 0.05 level (Table 5).

None of these clonal cell lines were found to produce a high affinity antibody, nevertheless, some anti-citrinin activity was detected. Because of the relatively low affinity of the antibodies produced, further development of techniques utilizing the products of the three clonal cell lines to actually detect citrinin were discouraged. However, since the procedures described in this paper have shown that it is possible to form citrinin specific monoclonal antibody, the task is delegated to future researchers to refine these techniques to achieve the end goal of a rapid, mab-based assay for citrinin.

Table 5. Clones of hybridoma 2B.2 assayed by ELISA for antibody to citrinin.

Clone	SWM/clt absorbance	SWM absorbance
2B.2.2.1	0.0717	0.0753
2B.2.2.2	0.0695	0.0710
2B.2.2.3	0.0740	0.0767
2B.2.2.4	0.0687	0.0723
2B.2.2.5	0.0712	0.0703
2B.2.2.6	0.0762	0.0770
2B.2.2.7	no growth	
2B.2.2.8	0.0782	0.0740
2B.2.2.9	0.0835	0.0723
2B.2.2.10	no growth	
2B.2.2.11	no growth	
2B.2.2.12	0.0870	0.0767
2B.2.2.13	0.1347	0.0793[a]
2B.2.2.14	0.1037	0.0886
2B.2.2.15	0.0956	0.0780
2B.2.2.16	no growth	
2B.2.2.17	0.1146	0.0817
2B.2.2.18	0.1093	0.0780[a]
2B.2.2.19	0.1277	0.0727[a]
2B.2.2.20	0.1017	0.0737
2B.2.2.21	0.0970	0.0817
2B.2.2.22	0.0933	0.0773

[a]Data are significant at the 0.05 level.

5. NEED FOR FUTURE STUDIES

Alternate Methods of Conjugate Production

It is important to the success of future endeavors to produce monoclonal antibodies to citrinin that a method be found to produce a more stable citrinin/protein conjugate. The most appropriate techniques may involve the mixed anhydride method utilizing EDPC (Chu et al., 1976). This procedure was successfully used to couple BSA to ochratoxin, a mycotoxin with reactive groups similar to those found on citrinin, in a conjugated form stable enough to produce high affinity antibodies upon immunization. Perhaps the utilization of a EDA-modified BSA would also serve to strengthen the stability of the conjugate and increase the mole binding ratio. The use of alternate protein carriers such as poly-l-lysine or human serum albumin should also be considered. These techniques should be evaluated in future studies and their applicability tested for producing a more appropriate citrinin immunogen.

Alternate Method of Mole Binding Ratio Determination

The HPLC method described for dtermination of the citrinin/BSA mole binding ratio is time consuming by the very nature of HPLC analysis. An interesting

comparison could be made using UV spectroscopy to analyze the dialysis buffers in addition to HPLC analysis. If UV spectroscopy was found to be reliable and sensitive, the more time consuming and costly HPLC procedure could be discontinued in future work. Any conjugates produced to be used as ELISA antigens (such as SWM/citrinin) should be analyzed to insure a high mole binding ratio as well.

Alternate Immunization Protocols

In future studies alternate immunization protocols should be evaluated in an attempt to maximize the immune response. Dose levels of the immunogen could be altered to determine what dosage results in maximum antibody production. The timing, frequency and total number of immunizations could also be maximized for efficiency. Different methods of immunization could also be compared such as subcutaneous verses intraperitoneal injections. All of these factors may have an influence on the immune response, hence an influence on desired hybridoma formation.

Alternate Cell Lines

This study may have been improved by using female RBF mice (unavailable at the time of this study) instead of male RBF mice. Female mice generally have

larger spleens, therefore more spleenocytes are available at the time of fusion. The strain of mouse used can also be a factor. This same experiment could be conducted using BALB/c, C3/HEJ or any of a number of other strains. In fact, this entire study could be conducted utilizing a different species of animal. Rats, rabbits, goats, swine and sheep are commonly used in such experiments.

The myeloma cell line used is also important. The myeloma cell line must be histocompatible with the species and strain of animal selected. The FOX/NY myeloma system was used in this study because it has the advantage of being genetically engineered such that any hybridoma that is formed must generate antibody to survive. This eliminates the necessity of screening out any unproductive hybridomas. It is possible that other myeloma cell lines could utilized.

Additional Analysis of Polyclonal Sera

The polyclonal sera produced by the mice in this study should be further purified and characterized. The affinity of the citrinin specific antibody present in the polyclonal sera could be determined and the antibody possibly incorporated into an analytical assay for citrinin determination. It is possible that, if

purified, the polyclonal anti-citrinin antibody could serve as a vaccine in the active immunization of animals against the effects of severe citrinin mycotoxicosis or in passive immunization as a prophylaxis against the effects of citrinin. This has been demonstrated with the mycotoxins zearalenone (Pestka et al., 1985) and T-2 toxin (Feuerstein et al., 1985).

Development of Assay for Citrinin Determination

Most importantly, future research should progress toward the goal of developing a rapid test for detection of citrinin. This will require the development of a hybridoma secreting high affinity antibody against citrinin and the incorporation of that antibody in a system suitable for citrinin detection. This paper has laid the groundwork for such research to proceed.

6. CONCLUSIONS

The results of this study indicate that:

1. Citrinin can be rendered immunogenic by simple conjugation to a protein carrier.

2. The citrinin/BSA conjugate is suitable for use in the formation of hybridomas secreting antibody specific for citrinin.

3. Hybridomas secreting anti-citrinin antibody may be cloned to produce a cell line secreting monoclonal antibody specific for citrinin.

It is not uncommon in this type of research to produce and screen many times the number of hybridomas assayed in this study before finding a suitable hybridomas secreting high affinity antibody. The scope of this project was limited by practical time and money constraints, nevertheless, it showed that production of citrinin specific mab is possible. Additional research in this vein will undoubtedly result in the development of a hybridoma secreting a citrinin specific antibody with a much higher affinity than the ones produced in this study. Additional research suggested by the results of this study is needed in several areas to make the development of a high affinity anti-citrinin mab a reality.

CHAPTER V

SUMMARY

This study was undertaken to determine if the low molecular weight mycotoxin, citrinin, could be made immunogenic through conjugation to a protein carrier, and to determine if an ELISA technique could be developed for detection of any subsequently produced anti-citrinin antibody. The last objective of this study was to determine if a hybridoma cell line secreting antibody specific for citrinin could be developed and cloned.

The results of this study indicate that, by simple direct conjugation to the protein carrier, BSA, citrinin can be rendered immunogenic so as to illicit an immune response in mice suitable for the formation of hybridomas producing mab specific to citrinin. The mabs produced in this study proved that the production of citrinin specific mab is possible. It is anticipated that future research will result in the production of high affinity anti-citrinin antibodies by variations of the procedures detailed in this study and

that a mab-based test for citrinin will become a reality.

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APPENDIXES

APPENDIX A

REAGENT AND MEDIA FORMULATIONS

Phosphate Buffered Saline (PBS)

	grams/liter (pH 7.2)
Na_2HPO_4 (7H 0)	2.16
KCl	0.20
KH_2PO_4	0.20
NaCl	8.0

Dissolve dibasic sodium phosphate first.
Autoclave for 20 minutes at 121°C.
Compounds should contain no Ca or Mg.

Hybridoma Complete Media (HCM)

5 ml	L-glutamine (50 ug/ml)
5 ml	amino acids
5 ml	vitamins
50 ml	NCTC 109 media
5 ml	oxaloacetate/pyruvate/insulin (OPI)
100 ml	fetal bovine serum (FBS)
5 ml	pen/strep antibiotics

Bring to total volume of 500 ml with Dulbecco's Modified Eagle Media (DMEM - with 2.25 g glucose in 500 ml). Add 11 ml HEPES buffer and adjust to physiological pH with NaOH. Filter sterilize.

Tris-ammonium chloride

Mix 90 ml ammonium chloride (0.16 M - 8.3 g/l) with 10 ml Tris (0.17 M - pH 7.65). Adjust pH to 7.2 with HCl. Filter sterilize.

Fusion mixture

Place 5.0 g of polyethylene glycol (PEG, MW=4000) in small (50 ml) flask. Add 0.5 ml dimethyl sulfoxide (DMSO) and 5.0 ml water. Cover flask with tin foil and place in larger beaker with boiling chips and enough water to cover the level of the liquid in the small flask. Cover beaker with tin foil. Autoclave at 121°C for 30 minutes. Dispense immediately into polystyrene tubes, 2.0 ml/tube. Keep sterile and seal with parafilm. Store in the dark at room temperature.

APPENDIX B

DATA AND CALCULATIONS

Sample calculation of mole binding ratio.

$$\frac{18.65\ \mu g\ cit}{264\ \mu g\ BSA} \times \frac{10\ \mu g\ BSA}{1\ g\ BSA} \times \frac{66000\ g\ BSA}{1\ mol\ BSA} \times \frac{1\ g\ cit}{10\ \mu g\ cit} \times \frac{1\ mol\ cit}{250\ g\ cit} = \frac{18.65\ mol\ cit}{1\ mol\ BSA}$$

ELISA of polyclonal sera pooled from three immunized mice compared to control (non-immune) mouse sera.

		ELISA antigen			
		SWM/citrinin		BSA/citrinin	
		1:16	1:32	stock	1:1
immune	1:50	0.210[a]	0.223	1.331	1.321
sera	1:100	0.171	0.195	1.389	1.378
normal	1:50	0.115	0.120	0.103	0.104
sera	1:100	0.110	0.106	0.092	0.102

[a]All data shown represent absorbance values at 405 nm on a model EL 310 EIA Autoreader.

VITA

John Eric Line was born to John and Martha Line in Pulaski, Virginia on February 13, 1963. He attended elementary and junior high school in Kingsport, Tennessee and graduated from Dobyns-Bennett High School in Kingsport in 1981. The following August he entered Carson-Newman College in Jefferson City, Tennessee and received a Bachelor of Arts degree in Biology and Chemistry in May, 1985.

In the fall of 1985 he accepted a graduate research assistantship at the University of Tennessee, Knoxville and began study toward a Master's degree. This degree was awarded in March, 1988.

He and his wife, Mrs. Renee Mimbs Line, were married on June 14, 1986. Mr. Line will be employed by the American Bacteriological and Chemical Research Corporation after graduation and will continue his studies at the University of Florida in Gainesville.