THE EFFECTS OF AFLATOXIN B_1 ON THE HEMATOPOIETIC SYSTEM

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THE EFFECTS OF AFLATOXIN B_1 ON THE HEMATOPOIETIC SYSTEM

An Abstract

Presented to

The Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Randy Steven Christophel

February, 1988

TO THE GRADUATE COUNCIL:

I am submitting herewith a thesis written by Randy Steven Christophel entitled "THE EFFECTS OF AFLATOXIN B_1 ON THE HEMATOPOIETIC SYSTEM." I recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in biology.

Robert J. Crews
Major Professor

We have read this
thesis and recommend its acceptance

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ABSTRACT

A standard sample of aflatoxin B_1 and a laboratory produced quantity of B_1 from the fungus A. flavus obtained from local agricultural feed were administered to male Sprague-Dawley rats to determine it's effect on the hematopoietic system. Although discrepancies were found in blood values, specifically WBC's, there is evidence that aflatoxin B_1 may have a direct effect on the hematopoietic tissue due to the resulting decrease in lymphocyte percentages and increasing monocyte percentages following toxin administration. In addition, a hyperplastic condition was observed in bone marrow examinations, and definite behavioral changes were noted in those animals administered aflatoxin B_1.

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INTRODUCTION

In the early 1960's researchers from around the world were gathered to investigate the etiological agent or agents responsible for a mysterious epidemic. This epidemic resulted in the death of thousands of domestic poultry and commercially raised fish in a variety of geographical locations. This disease was tentatively termed "Turkey X" due to its effect on domestic turkeys in Britain (Blount, 1961). The disease was characterized by loss of appetite, lethargy and a weakness of the wings, followed by death within a week. Postmortem and histological examinations revealed liver hemorrhages and necrotic lesions due to degeneration of the liver parenchyma cells and extensive proliferation of the bile duct epithelium cells (Blount, 1961). Simultaneous reports by commercial fish hatcheries throughout the United States indicating an outbreak of trout hepatoma due to unknown causes were also investigated (Wolf and Jackson, 1963).

Similar outbreaks were reported in Kenya and Uganda where ducklings and pheasants demonstrated the same characteristic symptoms as those mentioned above (Asplin and Carnaghen, 1961). Preliminary examination of suspect feed resulted in negative traces of plant toxins, insecticides, solvents, or alkaloids (Blount, 1961). Further inquiries noted a proximity of the

diseased animals within an 80-100 mile radius of London with one isolated feed mill supplying this area. Investigations found that 80% of the reported cases had dealt directly with this mill. The feed was analysed and brazilian groundnut, a component of the feed, was found to contain what was believed to be fragents of fungal hyphae (Asplin and Carnaghen, 1961). The fragents were later identified as that belonging to the species Aspergillus flavus Link ex Fries (Sargeant et al., 1961b). Purification of the feed revealed the presence of a substance considered to be toxic. The toxin was isolated by paper chromatography on Whatman No. 1 paper using n-butanol - 5% acetic acid as developer. The chromatography process resulted in a single spot at a R_f value of 0.7 emitting a bright blue fluorescence under ultraviolet light (Sargeant, 1961a). Verification was done by the extraction procedures mentioned above on cultures of A. flavus isolates in Czapek agar. Ingestion of the isolated material produced the characteristic symptoms of the disease in young ducklings. The newly discovered toxin was given the name aflatoxin (Sargeant et al., 1961b).

In the United States, cottonseed, an ingredient in feed rations used at trout hatcheries, was found to be the component associated with the etiologic agent in the outbreak of trout hepatomas (Wolf and Jackson, 1963). Further research revealed the presence of aflatoxin in the cottonseed meal (Jackson et al., 1968; Sinnhuber et al., 1965).

As chemical assays of aflatoxin continued, it was found that the toxin was composed of three additional components each revealing characteristic R_f values and fluorescence under UV light. Nesbitt et al. (1962) discovered the presence of a green fluorescent spot at R_f value 0.6 in addition to the blue spot at R_f 0.7. The two toxin components were labeled aflatoxin G and B respectively. Aflatoxin B was assigned the chemical formula $C_{17}H_{12}O_6$ and aflatoxin G the formula $C_{17}H_{12}O_7$ using elemental analysis and mass spectrometric determinations. Hartley et al. (1963) were the first to report the isolation of all four components on silica gel chromatoplates using chloroform: methanol (98:2) as the developing solvent. They termed the components B_1, B_2, G_1, G_2 according to decreasing R_f values and fluorescent color. Aflatoxin B_1 and G_1 were found to be the two components reported by Nesbitt in his research.

Aflatoxin is now known to be a toxic metabolic by-product of Aspergillus flavus, but numerous researchers have found that aflatoxin production is not restricted to A. flavus. Other fungi known to produce aflatoxins are Aspergillus oryzae (Basappa et al., 1967), A. parasiticus, (Codner et al., 1963), A. niger, A. wentii, A. ruber (Kulik and Holaday, 1967), and A. ochraceus (Van Walbeek et al., 1968). However, it is A. flavus that has dominated aflatoxin research and is generally found in higher percentages in contaminated products than the fungi mentioned earlier.

A. flavus has been studied in the deterioration of stored wheat, corn, rice, barley, bran, flour and soybeans (Semeniuk, 1954; Christensen, 1957). Other commodities later found to support A. flavus growth and aflatoxin production are egg noodles, cheese, condensed and powered milk, hazelnuts, walnuts, poppyseeds, coconuts, apple-juice, paprika, potato products, smoked bacon, dried peas, beans, lentils, plums, apple slices, peaches and figs (Frank, 1966).

Factors influencing maximum production of aflatoxin on natural substrates and in culture are temperature, time, aeration and moisture content. Temperature and moisture in particular are the two most important factors involved in the invasion of A. flavus and

production of aflatoxin on grain in storage bins or silos. Dickens and Pattee (1966) reported aflatoxin production in two days at moisture contents between 15% and 30% at 32.2° C and in four days at moisture content between 20% and 31% at 21.1° C in freshly dug peanuts. Diener and Davis (1966) observed optimal temperature and time for aflatoxin production by A. flavus on sterilized peanuts in culture flasks to be 25° C and seven to nine days. At 30° C, it took five to seven days for production and eleven to thirteen days at 20° C.

Extensive research has also been conducted involving aflatoxin degradation and microbial interaction. Schroeder and Ashworth (1965) and Wildman et al. (1967) have reported that fungi in competition with A. flavus and various substrates have degraded aflatoxin and might be responsible for smaller quantities of aflatoxin produced while competing for nutrients within the substrate. A decline in aflatoxin concentration after peak yields in culture were reported by Diener and Davis (1966). They concluded that degradation of aflatoxin correlated with mycelial lysis from high agitation rates and high aeration conditions. A. flavus isolates that formerly were not aflatoxin degraders were induced to do so by lysis. Adversely, Ciegler et al. (1966b) studied 1000 microoganisms including yeasts, molds, bacteria,

actinomyces, algal and fungal spores for their ability to degrade aflatoxin and only Flavobacterium aurantiacum was found to remove aflatoxin B_1 irreversibly from a nutrient solution.

Diseases involving the ingestion of aflatoxin have been studied and reported throughout the world. Reports as early as the 19th century of diseases caused by the consumption of discolored rice were recorded in Japan (Newberne, 1974). For decades, records of both man and animals infected with alimentary toxic aleukia shown to be caused by overwintered wheat have been noted. Wogan and Newberne (1967) have shown that levels as low as 0.015 parts per million (ppm) in the diet will induce a high incidence of hepatic carcinomas. Also studies have involved aflatoxin induced carcinogenesis in guinea pigs (Patterson et al., 1962), ducklings (Carnaghan, 1965), dogs (Newberne et al., 1966) and monkeys (Cuthbertson et al., 1967).

Researchers have found that most breeds of chickens are less susceptible to aflatoxin than other domestic and wild fowl species (Asplin and Carnaghan, 1961). A series of clinical and histopathological changes were reported in a field outbreak of aflatoxicosis in swine where the carcasses were found to be jaundiced and gross hemorrhages were observed throughout the body parts

(Loosmore and Harding, 1961). Lactating cows have shown a marked reduction in milk production following consumption of contaminated feed (Allcroft and Lewis, 1963). The death of three out of six suckling calves from a west African breed maintained on a diet of 1.5 mg of aflatoxin B_1 per day for four months was reported (Calvert et al., 1966).

Research involving dose response of aflatoxin, relating to mammalian consumption, has been concentrated on the laboratory rat. Widespread experimental techniques and a variety of aflatoxin quantities have been used to replicate the food chain process. As noted earlier, research has focused on the hepatic region due to the complications that have resulted from the effect of toxins in this body area. Wogan and Newberne (1967) reported the presence of hepatocellular carcinomas in 18/22 male Fischer rats 35-41 weeks following continuous administration of 1.0 ppm in the diet. Time, quantity and form of exposure of aflatoxin to the animal have all been studied extensively in connection with the formulation of hepatic carcinomas and other related abnormalities of the liver. A reflection of the hepatic disorders produced in rats has established a new trend in research procedures involving human liver diseases. Chaves-Carballo et al. (1967) reported finding a fifteen

year old girl exhibiting clinical and pathological features of Reye-Johnson syndrome with quantities of aflatoxin within her liver. Another case involved occupants of an isolated house which was found to contain high levels of A. flavus and A. parasiticus. Four cases of leukemia were reported among the three families residing in the house. The fungi were later found to produce large quantities of aflatoxin (Wray and O'Steen, 1975). Diagram I is a schematic representation of the interrelationships that exist between man, animals and aflatoxin B_1.

Although the core of aflatoxin research has concentrated on the hepatic region, investigations concerning less specific abnormalities due to aflatoxin ingestion have taken place. One area that has been studied less extensively is the effect aflatoxin has on the hematopoietic system. Tung et al., (1975) disclosed that chickens developed an anemia when fed measured doses of aflatoxin in the diet. The anemia was characterized by significant reductions in hemoglobin, packed cell volume and erythrocyte count. They reported that total leucocytes were increased three fold and the increases were due specifically to the neutrophils while basophils, lymphocytes and monocytes were reduced. It was also noted that eosinophils were found to be

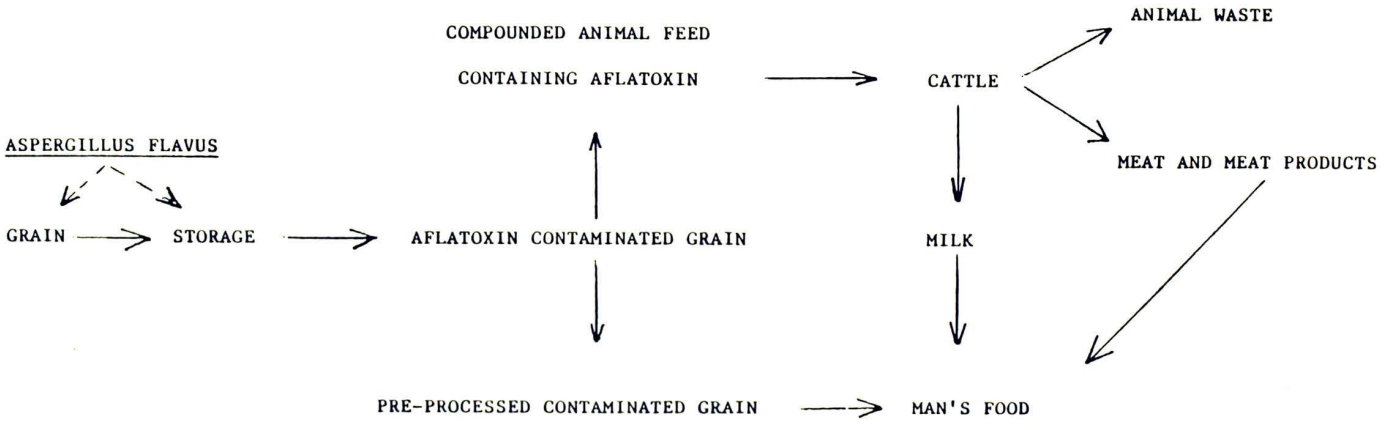

DIAGRAM I. INTERRELATIONSHIPS BETWEEN MAN, ANIMAL AND AFLATOXIN B_1. ADAPTED FROM PATTERSON, 1977

unaffected. Opposition to this study was reported by findings that there were not any significant changes in erythrocyte and leucocyte counts or hemoglobin determinations in ducklings (Juskiewiez et al., 1967). Wannop (1961) reported a case in which turkeys suffering from aflatoxicosis experienced an increase in neutrophils and monocytes while lymphocytes and erythrocytes were decreased. Pigs given diets containing aflatoxin were found to have an increase in leucocytes and erythrocytes (Harding et al., 1963). Sisk et al., (1968) and Garret et al., (1968) did not find any significant changes in hemoglobin or packed cell volume in pigs and negative affects of blood cell values in beef steers respectively when aflatoxin was consumed. A number of questions and uncertainties have therefore bordered the research in this area.

The objective of this study was to determine the various blood parameters of male Sprague-Dawley rats following ingestion of predetermined levels of aflatoxin B_1 in a daily diet.

METHODS AND MATERIALS

Samples of wheat, corn, cornmeal and soybean were collected from area storage bins and silos in the Montgomery County, Tennessee, vicinity. Storage time ranged from three and one-half months to ten months. Feed samples were also acquired from the Montgomery County Agricultural Department and various feed companies within the county.

Two forms of media were used to promote fungal growth: Sabaraud-Dextrose and Cornmeal agar. Sabaraud-Dextrose agar (32.5 g) was dissolved in 500 mL of distilled H_2O in a 1000 mL Erlenmeyer Flask. The ingredients were heated into a uniform solution and boiled for one minute. The agar was poured into two equal amounts in separate 2500 mL culture flasks and autoclaved for fifteen minutes at eighteen p.s.i. and cooled. A similar procedure was used to prepare cornmeal media, however 17 g of agar media were dissolved in 1000 mL of distilled H_2O. Additionally the cornmeal agar was separated into four 2500 mL culture flasks. The culture flasks were plugged and stored overnight in an air-flow hood to solidify.

One hundred grams of each feed sample were weighed and placed in 250 mL sterilized beakers. The samples were soaked for one hour in 75 mL of sterile H_2O.

Under a sterile hood the unabsorbed H_2O was retrieved from the feed samples and was poured into the culture flasks containing the Sabaraud-Dextrose and cornmeal agar until a thin layer covered the agar completely. The culture flasks were plugged with foam rubber stoppers and incubated at room temperature for observation of growth. Ten slant tubes each of Sabaraud-Dextrose and cornmeal agar also were inoculated with one mL of the collected spore suspension.

The incubation period was continued for ten days with daily observations for fungal growth. After the tenth day the cornmeal cultures showed a high proliferation of the species Rhizopus. Sabaraud-Dextrose cultures revealed a number of minute colonies which appeared to have Aspergillus spp characteristics. However, Rhizopus had grown over these colonies and morphological determinations for identification were not possible.

A second series of cultures were prepared using Sabaraud-Dextrose agar only. Ten 1000 mL culture flasks were set up according to the same procedure mentioned earlier. A sample of cornmeal containing a known quantity of aflatoxin was obtained from the Murray State Breathitt Veterinary Laboratory located in Hopkinsville, Kentucky. This sample was also used in the experiment.

One revision was made in this procedure: instead of 100 g of feed, only 25 g were used and soaked in 50 mL of sterile H_2O.

After four days at 37° C, six out of ten cultures revealed numerous fungal colony growths. To eliminate possible contamination by Rhizopus if the incubation time continued, the resulting growth was sub-cultured to Sabaraud-Dextrose agar plates. Using aseptic techniques, colonies were picked and streaked for isolation. The four remaining culture flasks were left undisturbed and continued incubation. The flask containing the sample of known aflatoxin revealed negative growth.

After four days of incubation at 37°C, isolated colonies were picked for identification. Morphological identification was determined using the slide culture technique (Koneman, Roberts, et al., 1978).

Using a surgical scalpel, two centimeter by two centimeter squares of Sabaraud-Dextrose agar were removed and placed on the sterile microscope slides. Each corner of the media square was inoculated with a loop containing fungal spores from an isolated colony. The squares were then covered with flamed cover slips and the slides were placed on sterile glass rods in the

prepared sterile petri dishes layered with sterile H_2O, covered and incubated at 37° C.

Three days after incubation had begun, mycelial growth and sporulation appeared adequate for identification. The cultures were removed from incubation and the cover slips were carefully separated from the agar squares. The cover slips were each placed on a separate microscope slide containing a drop of lactophenol-aniline blue stain. The agar squares were discarded into a 5% solution of phenol, and the remaining microscope slides were treated with a drop of lactophenol-aniline blue and covered with a clean slip and permitted to dry.

Microscopic examinations using oil immersion techniques allowed structural identification of Aspergillus niger, A. clavatus, A. flavus and A. fumigatus. All four species were transferred to fresh 2500 mL culture flasks containing Sabaraud-Dextrose agar. The flasks were incubated at 37° C for maximum growth potential and toxin production experiments. Although A. flavus was the targeted species, the researcher was interested in finding possible toxin production characteristics of the remaining three fungal species.

After five days of incubation, colony growth was sufficient to begin toxin experiments. A total of twelve 50 g grain samples was prepared. Four samples were prepared from each of three grains; corn, wheat and soybean. The samples were soaked in warm tap water for one hour, transferred to 250 mL Erlenmeyer flasks and plugged. The flasks were then autoclaved and cooled.

The four culture flasks containing the fungi were each washed with 20 mL of sterile distilled water to create a spore suspension. The spore suspension was then retrieved from the flasks and five mL of the suspension was inoculated onto each of the grain samples and the samples were incubated at 37° C.

Seven days after incubation had begun, mycelial growth was abundant enough to begin preparations for toxin extraction. The procedure used for extraction was the same method used at the Breathitt Laboratory with a few revisions introduced.

Twenty mL of 4% KCl and 180 mL of CH_3CN were mixed in a high speed blender containing 50 g of contaminated grain sample and blended at high speed for two minutes. The blended material was then filtered into three 250 mL beakers through Whatman #3 filter paper. The filtered solution was then transferred into a 250 mL separatory funnel. To extract fat contents 50 mL of

petroleum ether was added and the funnel was shaken vigorously by hand for fifteen seconds. The bottom layer was then retrieved and placed in a second separatory funnel where the fat extraction procedure was repeated. Approximately three grams of cupric carbonate were added to the fat extracted solution and transferred to a 500 mL beaker containing a gel composed of 170 mL of 0.2N NaOH and 30 mL of 10% Ferric Chloride. The contents were hand stirred for one minute and the slurry was again filtered through Whatman #3 filter paper into three separate 250 mL beakers. The filtered solutions were then combined into a 250 mL separatory funnel for final toxin extraction. Fifty mL of chloroform were added to the separatory funnel and shaken vigorously for one minute. The bottom layer was retrieved into a 400 mL beaker and the chloroform extraction was repeated. Following the second extraction, an ample amount of anhydrous sodium sulfate was added to the chloroform extracted solution to remove any remaining aqueous components. This solution was poured off into a 250 mL beaker and the beaker was placed in a flow through hood to allow the chloroform to evaporate overnight.

Observations the next day revealed an accumulation of a clear, crystallized material on the bottom of the

beaker. Using a sterile eye dropper, two drops of a Benzene:Acetonitrile (98:2) solution were added to the crystallized material. Once dissolved, the solution was inoculated onto TLC plates (Analtech, 250 ug silica gel plates, 47511) with one mL pipettes and placed into an airtight TLC chamber containing a solvent composed of Toluene: Ethyl Acetate: Formic Acid (90:45:15). After appropriate migration, the plates were removed and dried in a flow-through hood and observed under ultraviolet light. Results were negative concerning any signs of aflatoxin production.

A second sample of cornmeal was obtained from the Breathitt Laboratory containing known amounts of aflatoxin with levels ranging from 100 to 200 parts per billion (ppb). Three 50 g samples were again prepared for culture growth. However, the flasks were placed in incubators at various temperatures; two at 25° C, two at 37° C and two at 45° C. At day four of incubation, flasks at the 37° C range showed accelerated growth compared to the other temperatures. At day six, the 37° C flasks had already experienced spore production whereas the 25° C flasks were beginning mycelial growth. The flasks maintained at 45° C had not shown any sign of mycelial growth.

On day seven, the flasks were removed from incubation and the contents were transferred to petri dishes containing Czapek-Dox agar which was found to be an ideal media for isolation and differential identification of A. flavus (Koneman, Roberts, et al., 1978). In addition, culture slides were also prepared using Czapek-Dox media.

Eighteen dishes were set up and streaked. Six each were placed in incubators at 25°, 37° and 45° C. Five days after incubation, cultures from all temperatures were transferred onto culture plates. Three days later the plates were observed under the microscope for morphological identification. The foot cell, a definitive characteristic of A. flavus was observed repeatedly throughout the investigation and preparations were once again begun for toxin production experiments.

A preliminary test was first initiated to determine optimal toxin production environmental conditions. Twelve 25 g sterilized feed samples (six corn, six wheat) were placed in 250 mL Erlenmeyer flasks inoculated with a 10 mL spore suspension of isolated A. flavus and plugged. Two each of corn and wheat were incubated at 24° C. One flask of corn and one of wheat were agitated four times a day by hand, the other two were left undisturbed. The same process was followed

for flasks at 37° C and 45° C. On day four, the stable flasks at 37° C had abundant mycelial growth but the feed had a matted appearance. The agitated culture at 37°C showed minimal growth. On day six, the undisturbed cultures at 37°C began sporulation and the agitated flasks at 37°C were matted. The stable cultures at 24° C were also heavily matted, whereas the agitated cultures at 24° C appeared to have a uniform growth with negative matting. Cultures at 45° C still showed no signs of growth.

On day seven, all cultures were removed for toxin extraction procedures. TLC tests revealed that at 45° C toxin production was negative. The two agitated cultures at 37° C resulted in trace amounts of toxin with wheat and no toxin for corn. The same results were found in the undisturbed cultures at 37° C. However, the two agitated cultures at 24° C resulted in an ample quantity of toxin produced. Comparison of the corn and wheat TLC plates under UV light indicated a higher fluorescence produced by the wheat sample over the corn which still maintained an adequate fluorescence. Stationary cultures tested from this temperature range resulted in trace amounts of toxin produced.

An aflatoxin standard was used (Aldrich, 10 mg crystals) to determine aflatoxin concentrations. A

10 mg aliquot of crystals was dissolved in 10 mL of Benzene:Acetonitrile to get one mg/mL or 1000 ug/mL concentration level. From this stock solution, descending concentration levels were obtained through dilution and the various concentrated levels were inoculated onto TLC plates and placed in the TLC chamber. Comparison of the fluorescent spots of each concentration level resulted in the development of an accurate scale with which to quantitate the concentrations of toxin produced in culture. In addition, a definite R_f factor was determined for the type of TLC plates used. Comparison of the toxin produced in the laboratory with the standard under UV light indicated a concentration level at 100-250 ug/mL for the wheat sample and 75-150 ug/mL for corn samples. The R_f factor was found to be 0.5.

Since the optimal environmental parameters for toxin production had been established, preparations for maximum toxin production levels were begun. Using the same sterile procedures as before 12 cultures of 100 g samples were prepared (six corn, six wheat). All twelve cultures were left undisturbed at 24^{o} C for 24 hours prior to agitation. Following 24 hours of stationary incubation, the flasks were placed on a horizontal agitator and continuous agitation was set at

240 strokes/minute. Three flasks were removed at the third, fifth, seventh and ninth days for toxin extraction to determine optimal production time for aflatoxin. These samples were then compared to standard plates for concentration readings. Flasks taken on the fifth and seventh days produced levels of 200-400 ug/mL of toxin in both corn and wheat. Samples from the third and ninth days were minimal upon comparison with the standard scale. Once concentration levels were determined, the aflatoxin was streaked onto TLC plates, allowed to migrate and dried. The toxin was identified by UV light, collected by scraping the gel containing the toxin into vials and refrigerated.

Separation of the aflatoxin from the silica gel powder was tried by using Benzene:Acetonitrile (98:2), and centrifuging at 3000 rpm for five minutes. The supernatant was drawn off and TLC tests were run. The tests proved a negative recovery attempt. Another series of recovery procedures were tried using Acetonitrile:Potassium Chloride (90:10 4% Potassium Chloride solution). The solution was centrifuged at 3000 rpm for thirty seconds and the supernatant was removed and inoculated on TLC plates. Results were found to be 98% successful in aflatoxin recovery.

A level of 50 ug/mL of aflatoxin B_1 was determined from literature (Hayes, 1978) as the dosage concentration to be administered to the test animals. The proper dilutions were made and the pellets to be used to feed the animals were counted out according to the appropriate period of testing, allowing for one pellet per day per animal. The pellets were then soaked in the Benzene:Acetonitrile (98:2) solution containing the aflatoxin for thirty seconds each. The pellets were then placed in a sterile hood to evaporate the Benzene: Acetonitrile (98:2) off the pellets. Twenty-four hours after placing the pellets in the hood, samples were run through the toxin extraction procedure to insure that the aflatoxin had adhered to the pellets. TLC tests showed the aflatoxin on each pellet at 50 ug/mL level and the test animals were prepared for testing.

An advanced shipment of ten male Sprague-Dawley rats (Sasco Inc.) were received and preliminary blood tests were begun to determine blood standards. Each animal was weighed daily in a live specimen basket scale and recorded.

Blood parameters determined included Red Blood Cell Counts (RBC), White Blood Cell Counts (WBC), Hemoglobin levels (Hb), Hematocrit Values (Hct), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and

Mean Corpulscular Hemoglobin Concentration (MCHC). Capillary blood was obtained by clipping the tip of the tail with a surgical scalpel blade and obtaining 25 uL of blood.

The blood parameters were determined by using a Clay Adams Company Ultra Logic 800. Following the procedure for capillary blood, the 25 uL were dispensed into a premeasured reservior containing 6.5 mL of buffered isotonic diluent with a resulting dilution of 1:260. The Ultra Logic uses a WBC-Hb port for counting WBC's and measuring Hb and an RBC-Hct port for counting RBC's and calculating the Hct. In addition, a built-in computer uses the RBC, Hb, and Hct values to compute the indicies (MCV, MCH and MCHC). The 1:260 dilution for second dilution was accomplished by using the diluter built into the instrument. It draws up 35 uL of sample and dispells it along with 9.1 mL of the isotonic buffered diluent. This produced a final dilution of 1:67,000. Three drops of hemoglobin lysing reagent were added to the first dilution and then both reserviors were introduced into the proper ports for counting. The RBC values of the rats were found to be higher than the counting range of the instrument. This meant that the second dilution had to be diluted further. This was accomplished by pipeting two milliliters of the dilution

into a clean reservior and adding two milliliters of diluent. The resulting RBC and Hct values had to be multiplied by two and the indices had to be manually calculated. This procedure was followed for all subsequent counts.

Blood smears were also made at the time of each collection. The smears were stained with Wright's stain for differential counting of WBC's, as well as morphological evaluation of all cellular elements.

The animals were housed independently in shoe-box type cages in a well ventilated, filtered room. Room temperature was maintained at 20° C and a 12 hour light-dark cycle was used. Water was taken ad lib and 30 g of feed pellets were given daily. Daily observations concerning appetite, water consumption and activity were recorded. At the end of the test period, the animals were sacrificed and femur bone marrow smears were made. Liver tissue was also removed and stored for future examinations.

A second shipment of twenty-five male Sprague-Dawley rats was obtained to be used for the experimentation processes of toxin ingestion and hematopoietic response. Ten of these animals were subjected to 30 g of daily feed containing a 50 ug/mL concentration level of standard toxin. These animals were labeled

standards A-J. A second group of ten received 30 g of feed daily containing 50 ug/mL of aflatoxin B_1 produced in the laboratory. This group was labeled APSU 1-10. The five remaining animals were labeled Control II (A-E) group and received a 30 g diet of feed that contained one pellet per day per animal with Benzene:Acetonitrile (98:2) evaporated off. Water was given ad lib and environmental conditions were identical to the preliminary experiment.

The animals were tested daily and the results recorded for blood parameters, weight results and daily activities. The test animals were sacrificed after 16 days and blood smears were taken along with bone marrow smears and liver tissue samples.

RESULTS

As illustrated in Table I, comparison of all four test groups revealed an elevated level of WBC's in the Standard and APSU B_1 aflatoxin groups. However, Control II test animals likewise experienced an elevation of white blood cells.

In contrast, Standard B_1 and APSU B_1 groups showed an average RBC value of $8.37mm^3$ and $8.33mm^3$ respectively. This is a slight reduction compared to the resulting value of $8.67mm^3$ Control I animals. Animals in Control II experienced an even more drastic decline with a $7.42mm^3$ RBC count.

With a decrease in RBC's, an expected reduction in hemoglobin concentration should be proportionately reduced. However, both groups receiving B_1 exhibited concentration levels higher than that of the Control I group. Control II showed the normal reduction in Hb levels in accordance with it's reduction in RBC's.

The same pattern was seen when hematocrit levels were taken. Standard B_1 animals revealed a level of 45.1% and those of the APSU B_1 group resulted in an average of 43.2%. Control I animals maintained a middle range of 42.2% while Control II dropped to 40.8%.

TABLE I

TOTAL DAILY BLOOD VALUE AVERAGES

GROUP	WBC	RBC	Hb	Hct	MCV	MCH	MCHC
Control I	7.5	8.67	16.4	42.2	50	19.0	38.9
Control II	12.5	7.42	16.1	40.8	55	21.6	39.5
Standard B_1	12.6	8.37	17.1	45.1	54	20.5	38.2
APSU B_1	12.1	8.33	17.2	43.2	52	20.6	39.8

Figures one through four give a daily average account of the four major blood parameter values tested among the four groups. Figure one shows the erratic pattern of WBC calculations among the three groups exposed to either Benzene:Acetonitrile (98:2) or aflatoxin B_1. Control I group experienced a consistent daily production level of WBC's in comparison to the others.

Figures two and three, which chart RBC and Hb levels respectively, exhibit a rather consistent scheme of production. Whether the effect of Benzene:Acetonitrile (98:2) kept RBC levels down within the B_1 was not determined.

As seen in the WBC illustration, Figure four reveals an inconsistent behavior in Hct percentage levels. However, this erratic behavior is exhibited in all four groups tested.

Blood differentials were calculated by microscopic examinations of blood smear slides taken daily. The slides were stored until all testing was completed and then examined. The findings of the examinations are listed in Table II.

Both Control I and Control II groups showed a consistent percentage of the characteristic white blood cells. Control II did experience an increase of 4.2% monocytes to 2.0% monocytes in Control I. This was the

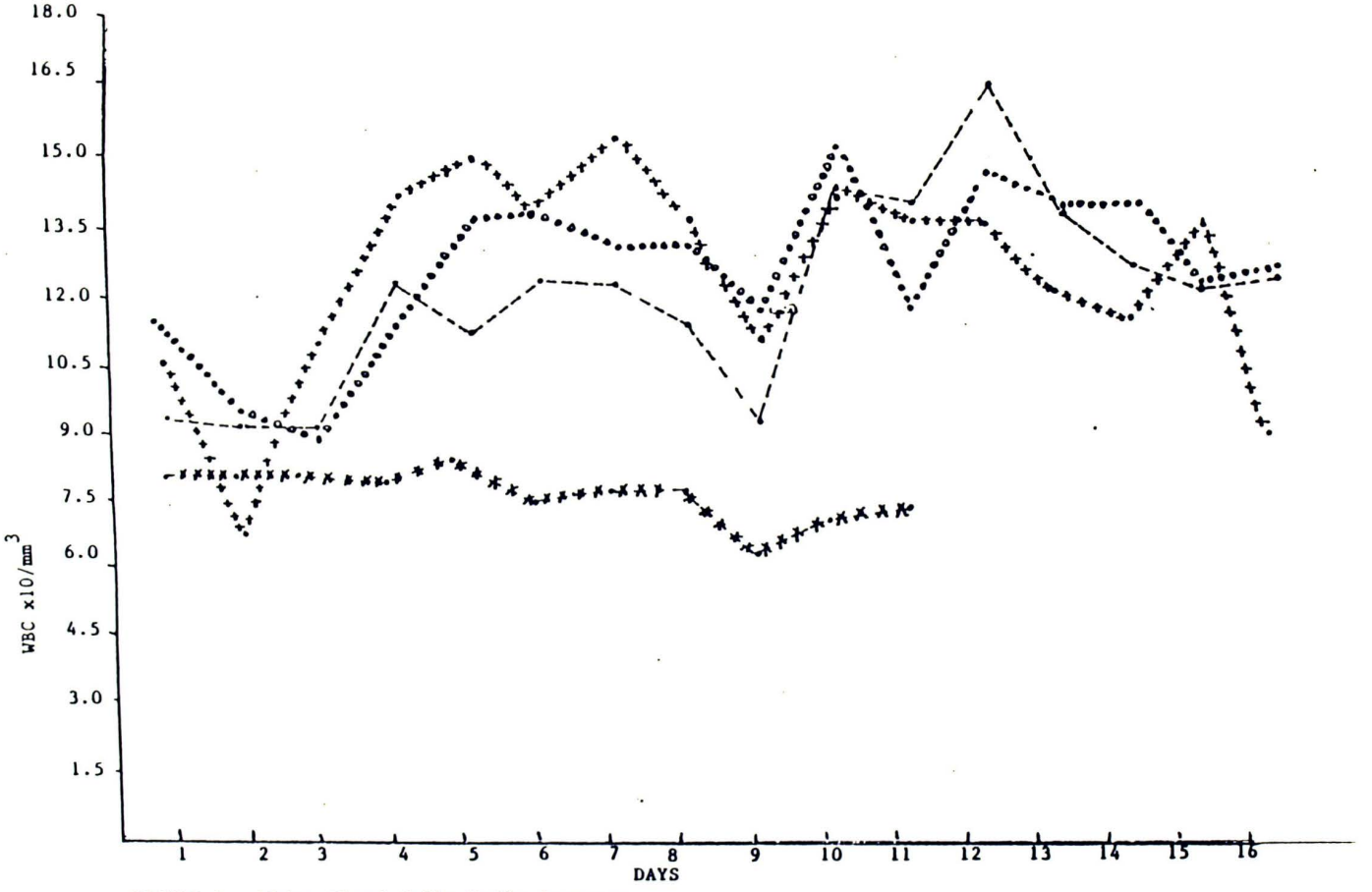

FIGURE 1. White Blood Cells Daily Averages

— APSU
Standard
Control I
Control II

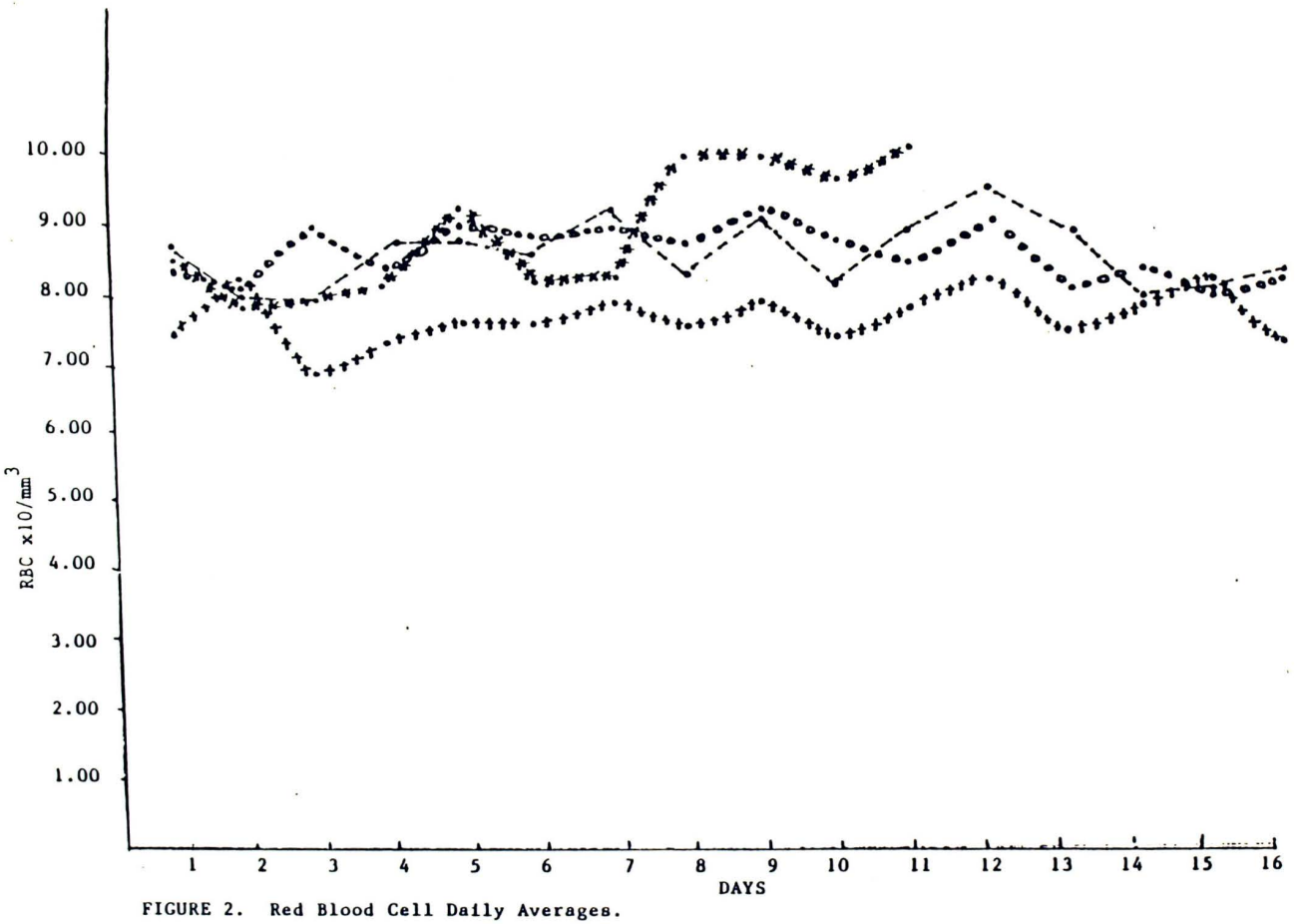

FIGURE 2. Red Blood Cell Daily Averages.

— APSU
•••Standard
###Control I
+++ Control II

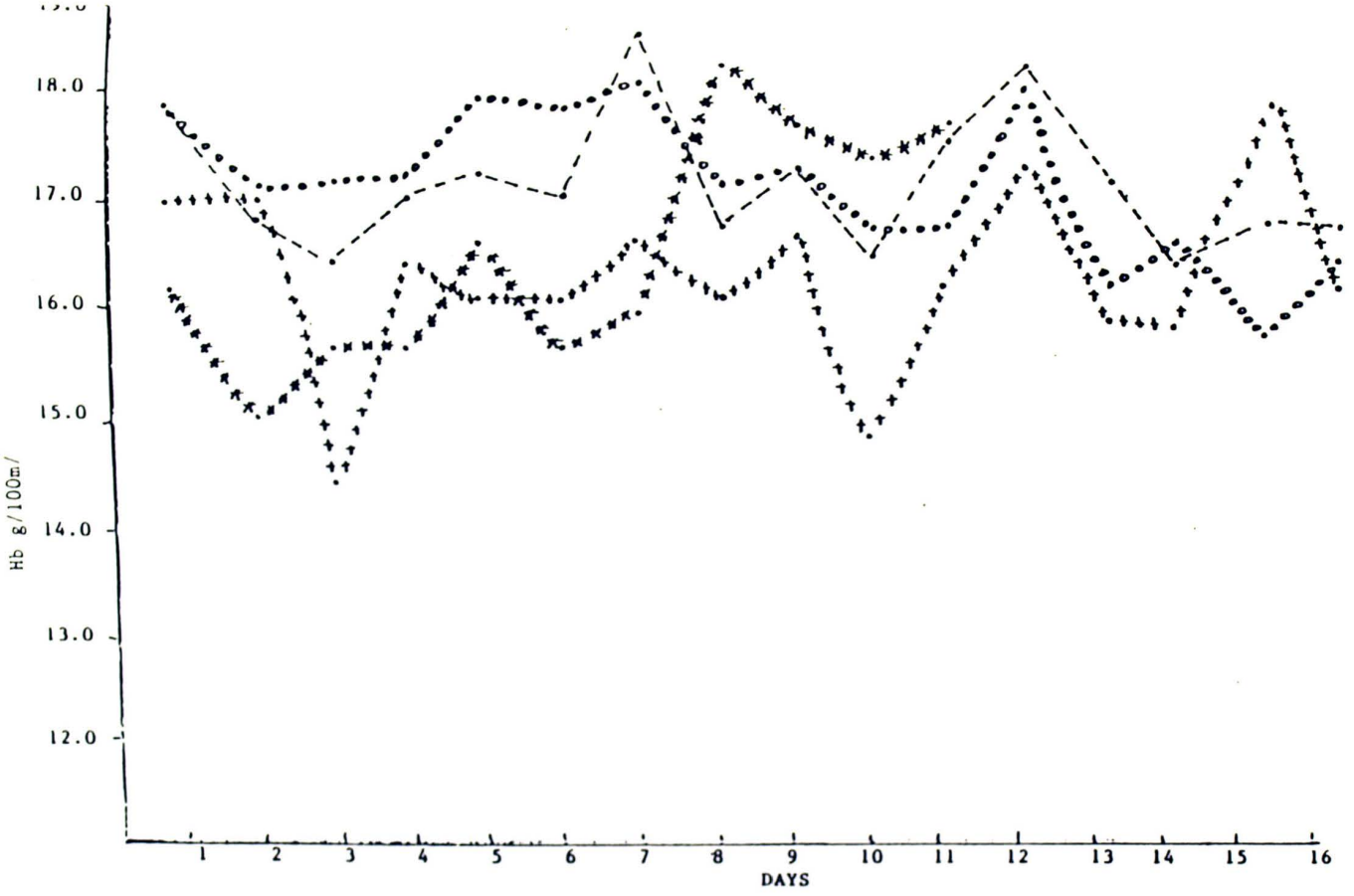

FIGURE 3. Hemoglobin Daily Averages.

— APSU
∘ ∘ ∘Standard
* * *Control I
† † †Control II

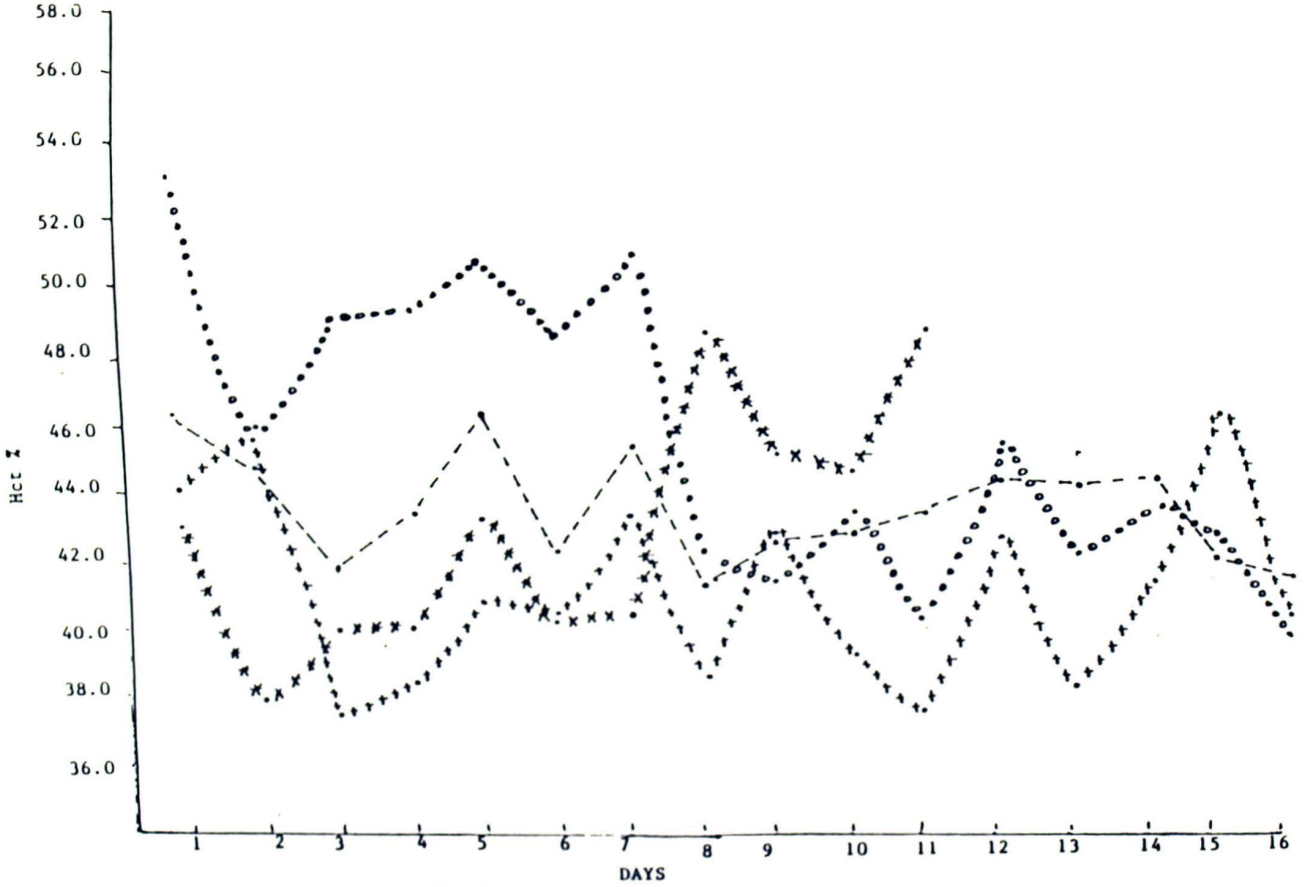

FIGURE 4. Hematocrit Daily Averages

— APSU
Standard
Control I
Control II

TABLE II. BLOOD DIFFERENTIAL TOTAL AVERAGES

GROUP	LYMPHOCYTES	NEUTROPHILS	EOSIONOPHILS	MONOCYTES
Control I	87.4	7.9	1.6	2.0
Control II	86.7	7.9	1.3	4.2
Standards	75.4	9.4	1.7	14.3
APSU	73.2	11.5	0.9	14.1

only change to be noted. Likewise, lymphocytes were reduced in the two groups exposed to aflatoxin B_1. Standard B_1 was found to have 75.4% lymphocytes while APSU B_1 was 73.2% per 100 cells examined. An even more drastic occurrence was seen in monocytes. An increase of a 14.1-14.3% range of monocytes in the B_1 groups from a sub 5% range in the control groups was exhibited.

Following the final day of testing, the animals were sacrificed and bone marrow smears were prepared. Upon examination of the smears from the animals receiving aflatoxin B_1, a hyperplastic condition was indicated. Due to the immaturity of the cells within the bone marrow, evidence as to the abundant rise in monocytes could not be verified.

Daily observation of the test animals revealed that the animals receiving aflatoxin B_1 in the diet appeared lethargic in behavior, and H_2O consumption radically increased as compared to those in the control groups. Appetite appeared not to be affected in any of the groups tested, therefore assuring the ingestion of the dissolved aflatoxin B_1.

DISCUSSION

Research involving the effects of aflatoxin B_1 has been virtually non-existent in relation to human contact or ingestion of the fungal metabolite. As noted earlier, aflatoxins have been mentioned in human diseases and maladies throughout history. Control of this toxin has been adequately maintained in North America as evidenced by the small number of cases reported. A review of the cases reporting aflatoxin in agricultural feedstuffs on file at the Breathitt Laboratory from 1977-82 shows that 81 samples (16/year) were found to contain high levels of aflatoxin B_1 (50-100 ppb), whereas in 1983 only 13 cases had been reported. There is still a need for further control of this known carcinogenic agent.

Reports of aflatoxin B_1 producing symptoms of aplastic anemia (Forgacs and Carll, 1962 and Tung et al., 1970, 1971) and hyperplastic conditions (Tung et al., 1975) have been noted and reviewed. Abnormalities of the hematopoietic system in connection with aflatoxins have been researched and reported. However, controversy seems to be the end result. To the present date, research involving aflatoxin and its effect on hematopoietic tissue has still only been superficially

done. Therefore, controversy will abound until more in depth research has been done on this topic.

The research presented within has done nothing to alleviate the controversies surrounding this area of study. One important factor has surfaced that needs further research. As noted in the METHODS AND MATERIALS section, the solvent Benzene:Acetonitrile was used to dissolve the B_1 crystals. It is possible that total evaporation of the Benzene:Acetonitrile solution had not taken place from the feed pellets given to the test animals. What effects the solvent in question had on the remaining blood cell parameters are speculation.

Review of blood differentials suggests that the aflatoxin B_1 did affect hematopoiesis. The decreases of lymphocytes and increases of monocytes by both groups administered B_1 and the parallel results of the control groups shows that hematopoiesis was affected. Bone marrow examinations also support peripheral blood findings. Examinations of bone marrow slides showed abnormal hypercellularity with an increase in immature blood cells, which was especially evident in the granulocytic series of blood cells. The presence of aflatoxin B_1 may also be the cause of a number of unexplained blood pictures observed during the research. In addition, the physical behavior demonstrated by the

animals receiving aflatoxin B_1 would give further support to accelerated hypercellularity due to aflatoxins.

Tung et al. (1975) reported a condition similar to this hypercellularity in his research. It was expressed as a hemolytic anemia due to increased leucocyte counts and marrow hyperplasia.

However, much of the research like that of Tung's has been mainly limited to the avian species and to a lesser extent to domestic animals. To the author's knowledge this is the first time that tests of this type have been used on rats. Therefore, a different criterion may result in the mammalian rat response to the invasion of aflatoxin as opposed to other test animals used in past research. In addition, it has been suggested that although aflatoxin B_1 is at present the most potent fungal toxin known to man, it may produce its effects in conjunction with other toxins such as rubratoxin, a toxic metabolite produced by Penicillium rubrum known to have physiological effects on various systems of the body (Edwards and Wogan, 1968).

Another factor that must be considered is that of time. From the observations of increased cytopoiesis in the bone marrow, this researcher feels that an increased amount of time must be allowed to accumulate consistent

results. Literature reviews indicate that test periods have been of a short duration which may account for the various discrepancies in other reports.

Although this study shows evidence that aflatoxin B_1 could be considered a causative agent in causing disruption of the hematopoietic system, this researcher feels that a second phase of experimentation must be undertaken using a solvent other than the Benezene:Acetonitrile solution to provide a more accurate account of the blood parameters.

SUMMARY

Evidence exists within this study that aflatoxin B_1 may exert an effect on the hemotapoietic system. Peripheral blood findings, along with observations of bone marrow hyperplasia support this conclusion. However, additional research needs to be done due to the unforseen factors involved with the solvent Benzene: Acetonitrile used to prepare the toxin for the animals diet.

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