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LYMPHOCYTE POPULATIONS AND THEIR RELATIONSHIP TO CELL-MEDIATED IMMUNE RESPONSES IN AFLATOXIN-TREATED GUINEA PIGS

Iowa State University PH.D. 1982

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Lymphocyte populations and their relationship to cell-mediated immune responses in aflatoxin-treated guinea pigs

by

Mary Ellen McLoughlin

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

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For the Graduate College

Iowa State University
Ames, Iowa

1982

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GENERAL INTRODUCTION

Aflatoxin is the best known among the mycotoxins because of its toxicity, carcinogenic potential, and worldwide occurrence in many feedstuffs. As commonly used, 'aflatoxin' refers to any one of several chemically related secondary metabolites produced by toxigenic strains of Aspergillus flavus and A parasiticus. The primary aflatoxins B_1, B_2, G_1, and G_2, of which B_1 is the most toxic, can produce hepatotoxic effects in numerous animal species.[1]

Wide variations in susceptibility to aflatoxin exist among species and individuals within a species.[2] Generally, young animals are more susceptible to aflatoxin than are adults.[1] At low concentrations, aflatoxin may lower an animal's resistance to infection without producing clinical signs readily referable to ingestion of aflatoxin. The true etiology of such secondary mycotoxicoses is often overlooked.[3]

In 1960, coincidental toxic events resulting in the mortality of thousands of turkey poults in England,[4] as well as in several other species elsewhere,[1] and an outbreak of trout hepatoma in the United States[5] focused international attention on a presumed contaminant of some ground grain meals. In 1961, a toxin was isolated, characterized chromatographically, and named aflatoxin.[6,7] Aflatoxin ingestion has been associated with field cases of salmonellosis and candidosis in poultry[8] and increased incidence of hepatocarcinomas in humans.[9] Aflatoxin has been shown to affect adversely specific and nonspecific immune responses in field and experimental situations.

Aflatoxin may reduce complement activity,[10] interferon production,[11] and phagocytosis.[12] Native resistance of animals to infection may be altered by consumption of aflatoxin. Increased susceptibility to infection with more severe disease sequelae was noted in chickens with candidosis,[13] coccidiosis,[14] and salmonellosis,[15] and in calves with fascioliasis.[16] In other models, the incidence and severity of aspergillosis in turkeys,[17] salmonellosis in chickens,[18] and paratuberculosis in hamsters[19] were not affected by aflatoxin. Similarly, aflatoxin impaired specific immunogenesis in some systems but not in others. Turkey poults vaccinated to Pasteurella multocida during a period of aflatoxin consumption were not adequately protected against challenge.[20] Aflatoxin-treated swine did not develop adequate immunity to Erysipelothrix rhusiopathiae.[21] However, aflatoxin had no effect on the ability of turkeys to develop immunity to Newcastle Disease Virus.[11]

Cell-mediated immune (CMI) processes appear to be more susceptible to toxic effects of aflatoxin than do immune responses involving primarily humoral antibody. In the study cited above,[20] the antibody response was normal in those turkeys which were unable to develop immunity to P multocida. Although antibody suppression has been observed in three studies,[22-24] most reports indicate that antibody production is refractory to any effects of aflatoxin except at levels which cause overt aflatoxicosis.[25] Increased levels γ globulin have been associated with aflatoxin ingestion[26] and are probably referable to the hepatotoxic effects of this mycotoxin rather than to a specific immune alteration. Increased levels of

γ globulin observed in other chronic hepatic diseases result from the inability of the liver to remove antigens before they reach antibody producing sites.[27]

Additional evidence that aflatoxin selectively impairs cell-mediated immune processes has accrued for several animal species.[25] Aflatoxin causes thymic involution,[28,29] depresses delayed type hypersensitivity,[23,30] lymphokine activity,[30] lymphoblastogenesis[30-32] and the graft versus host response.[23]

Animal species vary widely in their susceptibility to aflatoxin. In the guinea pig, 0.070 mg/kg/day aflatoxin B_1 equivalents (B_1 eq) administered orally for three weeks caused diminished skin test responsiveness.[30] Lymphokine activity (migration inhibition factor) and lymphoblastogenesis were also reduced in this study, but the differences were not significant, perhaps owing to individual variation, small sample size and limited sensitivity of the assays employed. Complement deficiency and reduced weight gains were apparent in guinea pigs which received a comparable dose of aflatoxin.[10]

A precise analysis of aflatoxin's mode of action on the cellular immune system has not been feasible due to limited technology and lack of knowledge of reactive metabolites. The observed suppression of cell-mediated immunogenesis and thymic involution suggests that aflatoxin may be selectively toxic for T lymphocytes. Reductions in lymphokine activity or production and lymphoblastogenesis favor a functional hypothesis to explain the defect in T lymphocytes caused by aflatoxin. Both MIF activity and

lymphoblastogenesis depend on active protein synthesis,[33] which is impaired by aflatoxin.[34] Delayed type hypersensitivity responses depend on a sensitized T lymphocyte and a nonspecific macrophage. Skin testing is useful because it is an *in vivo* assay of delayed type hypersensitivity. However, it is impossible to measure the relative contribution of the sensitized T lymphocyte and the macrophage to the skin test response. Thus, reductions in skin test responsiveness in aflatoxin-treated guinea pigs may reflect a quantitative or qualitative change in T lymphocytes or macrophages, or impaired interactions between the two cell populations.

Guinea pigs are uniquely suited for an analysis of aflatoxin's effect on cell-mediated immune processes because: (1) they are moderately to highly susceptible to aflatoxin;[35] (2) it is economically feasible to sample a large population of guinea pigs; and (3) they provide a well-characterized model for studies of cell-mediated immunity.[36] The present studies attempted to define a threshold of susceptibility for CMI responses (as measured by delayed-type hypersensitivity) of aflatoxin-treated guinea pigs. Guinea pigs receiving graded doses of aflatoxin were sensitized to *Nocardia asteroides* during aflatoxin consumption. Skin test responses were recorded at the end of the experiment. Weight gain and concentrations of serum bile acids were recorded and used to relate hepatotoxic effects of aflatoxin to immunosuppressive effects. The results of this investigation are described in Section I. This dose-response data was used as a guideline in subsequent experiments which examined the nature of aflatoxin-induced immunosuppression by (1) passive transfer of immunity and (2) enumeration of lymphocyte populations.

Bloom and Chase prepared a thorough monograph on passive transfer of delayed type hypersensitivity in the guinea pig.[36] Passive transfer of delayed type hypersensitivity is an in vivo assay in which sensitized T lymphocytes from a 'donor' animal are transferred to a nonsensitized 'recipient' animal. The transferred T lymphocytes provide a signal in the form of a lymphokine[37] to recipient macrophages which comprise the major cellular component[36] at the skin test reaction site. As little as 0.06% sensitized lymphocytes, when mixed with nonsensitized lymphocytes, are sufficient to elicit a positive skin test response in a recipient animal.[38] Since the recipient skin test response is dependent on the numbers and viability of transferred sensitized lymphocytes,[36] passive transfer experiments afford the investigator an opportunity for standardizing delayed type hypersensitivity events within limits of individual variation.

Using a passive transfer model in Section II, experimental donor and recipient groups were established to test the relative contributions of sensitized T lymphocytes and macrophages from aflatoxin-treated guinea pigs to the resultant skin test reaction. Peritoneal exudate cells (PEC) from nontreated or aflatoxin-treated, sensitized guinea pigs were injected into nontreated or aflatoxin-treated, nonsensitized recipients. The numbers and viability of PEC transferred were standardized in each experiment.

Aflatoxin ingestion may cause a quantitative change in lymphocyte populations which results in immunosuppression. Thus, determination of T and B lymphocyte populations in aflatoxin-treated guinea pigs was a logical correlate to the aforementioned functional studies. Currently, identi-

fication of guinea pig B and T lymphocytes is based, respectively, on the detection of surface immunoglobulin[39] or the formation of heterologous red blood cell rosettes.[40] These techniques are tedious, require purified populations of lymphocytes and yield highly variable results.

Monoclonal antibodies with specificity for guinea pig T lymphocytes are now becoming available.[41] While their specificity is unsurpassed, identification of T or B lymphocytes using antibody has many technical complications. A purified lymphocyte population is required, identification of cells must be made using phase contrast or fluorescence microscopy, immunofluorescence fades, and distinction must be made between unstained monocytes and lymphocytes. Contrast a bacterial binding assay developed by Teodorescu et al[42] for the identification of human and murine lymphocytes in which the property of some lymphocytes to bind spontaneously to certain bacterial strains is exploited. The bacterial binding assay employs whole peripheral blood. One lymphocyte typically binds twenty or more organisms which facilitates its identification and distinction from other cell types in stained blood smears using light microscopy. In humans, the populations of lymphocytes identified by bacterial markers have functional analogy to lymphocyte populations identified by cytoxic activity, mitogenic response and allogeneic reactivity.[43,44] Bacterial binding as a method for identifying lymphocyte subpopulations is the subject of a recent review.[45]

Section III describes experiments in which numerous strains of bacteria and yeasts were screened to identify strains which consistently bound a percentage of lymphocytes in a number of guinea pigs. Potential lympho-

cyte markers were studied further to determine which lymphocyte populations were binding the bacteria.

In Section IV, T and B lymphocytes from nontreated and aflatoxin-treated guinea pigs were counted using bacterial markers and conventional direct and indirect immunofluorescence assays.

The four sections of this dissertation represent papers submitted for publication to the American Journal of Veterinary Research. Each section, as well as the introduction, has been prepared using the format of that journal. These papers represent original research investigating the means by which aflatoxin causes suppression of cell-mediated immune responses in guinea pigs.

SECTION I. EFFECT OF GRADED DOSES OF AFLATOXIN ON DELAYED TYPE HYPERSENSITIVITY AND WEIGHT GAIN IN GUINEA PIGS

INTRODUCTION

Aflatoxin inhibits cell-mediated immune processes[1,2] but does not alter antibody production except at high levels of consumption.[2,3] Thymic involution,[4] reduced delayed type hypersensitivity (as detected by skin test responsiveness and lymphokine activity),[1] reduced lymphoblastogenesis,[1,5,6] and diminished graft vs host response[2] are caused by aflatoxin in a variety of animal species.

In guinea pigs, ingestion of a moderately high level of aflatoxin (approximately 0.070 mg/kg/day aflatoxin B_1 equivalents) over a three week period caused a significant reduction in skin test responsiveness. Other indices of cell-mediated immune responsiveness, including lymphokine activity and lymphoblastogenesis, were marginally affected,[1] reflecting perhaps the limited sensitivity of the assays employed and the biological variation of response by treated principals.

The experiment described herein was initiated to define the level of aflatoxin ingestion at which cell-mediated immune responses in the guinea pig are impaired and to compare it to the level at which reduced weight gain, a consistent change caused by aflatoxin ingestion, becomes apparent.

MATERIALS AND METHODS

Animals--Female guinea pigs (400-450 g) from a closed colony at the National Animal Disease Center (Ames, IA) were used. For each of three trials, they were randomly distributed into experimental groups of ten animals per group and given water and pelleted guinea pig ration (Teklad, Monmouth, IL) ad libitum. Total body weight was recorded for each animal bi-weekly (see Table 1).

Aflatoxin--A partially purified preparation of aflatoxins (PPA) was used. Densitometric analysis of the preparation by thin layer chromatography under UV light showed that it contained 37.83% B_1, 1.55% B_2, 18.48% G_1, and 0.65% G_2. By considering the relative contribution of each aflatoxin to the overall toxicity,[7,8] the preparation was assigned a value of 47% B_1 equivalents (B_1 eq). PPA was dissolved in chloroform and dispensed into gelatin capsules (Size #5, Lilly Pharmaceutical, Indianapolis, IN). Capsules were administered daily per os for three weeks according to the dose schedule presented in Table 1. Control animals received empty capsules.

Delayed type hypersensitivity--Aflatoxin-treated and nontreated guinea pigs were sensitized to Nocardia asteroides as previously described.[9] Briefly, heat-killed organisms (preserved with merthiolate at a final concentration of 10^{-3}) were ground in light oil (Bayol-55, Humble Oil Co., Houston, TX) and suspended in same to a final concentration of 0.4 mg/ml. On day 3 of aflatoxin treatment, 0.1 ml of this suspension was injected intradermally in four locations on the abdomen of each animal. On day 24,

all sensitized animals and nonsensitized controls were skin-tested by intradermal injection (0.1 ml) of homologous N asteroides culture filtrate sensitin.[10] Induration (measured by calipers in millimeters) was recorded at 24 hours:

Induration = reaction skin thickness - normal skin thickness

Histopathology--Liver tissue and tissue excised from the skin test sites of representative animals were fixed in 10% buffered formalin, and stained with hematoxylin and eosin.

Serum concentrations of bile acids--On day 24, guinea pigs in Trial I were anaesthetized with carbon dioxide and bled by cardiac puncture. Ten ml of blood was collected and allowed to clot at room temperature. Serum was collected and stored at -70°C until assayed. Concentrations of glycocholic acid and glycodeoxycholic acid were determined by radioimmunoassay according to the directions provided with the assay kit (Nordiclab Uusitkatu 92, SF-90120, Oulu 12, Finland).

RESULTS

The results of three trials of the experimental design (Table 1), comparing weight gain and skin test responsiveness of aflatoxin-treated and nontreated guinea pigs, are shown in Figs 1 and 2. Generally, aflatoxin caused reductions in weight gain and skin test responsiveness in a dose-related manner.

Analysis of variance[11] indicated that the pretreatment weights were similar for all 7 groups of guinea pigs within any given trial. Following daily oral administration of PPA for three weeks, groups 4, 6, and 7 in trial I, groups 4, 5, 6, and 7 in trial II, and groups 5 and 7 in trial III showed reduced weight gain when compared to treatment control groups 1 and 2 ($P < 0.05$). In all three trials, reduced skin test responses were noted at and above dose level 6 (0.040 mg/kg/day B_1 eq) (Fig 1). Reductions in weight gain paralleled changes in skin test response, but were evident at lower dose levels (at and above 0.020 mg/kg/day B_1 eq) (Fig 2).

Twenty-four hours after the skin test for sensitivity to N asteroides, no necrosis was visible at the skin test site. Histologic examination of representative tissue sections excised from skin test sites revealed a predominantly mononuclear cell infiltrate. Diminished skin test responsiveness was observed in groups 6 and 7 for all trials ($P < 0.05$) (Fig 2). Wide variations in skin test response were noted among individuals within aflatoxin-treated groups. In groups 5, 6, and 7 (Trials I, II, III), the skin test response was below the group mean for well over half of the animals in the group.

Table 1 Experimental Design

Groups	Sensitization	Aflatoxin (mg/kg/day B_1 eq)
1	Nonsensitized	0.000
2	Sensitized	0.000
3	Sensitized	0.010
4	Sensitized	0.020
5	Sensitized	0.030
6	Sensitized	0.040
7	Sensitized	0.070

There were 10 animals per group. All animals were weighed on day 0 and biweekly thereafter. Aflatoxin was administered daily *per os* from day 0 to day 24. Animals in groups 2-7 were sensitized to *N asteroides* on day 3. They were skin tested with homologous sensitin on day 24 and induration was measured on day 25. Serum was collected on day 25 from all animals in Trial I for determination of levels of glycocholoic and glycodeoxycholic acids.

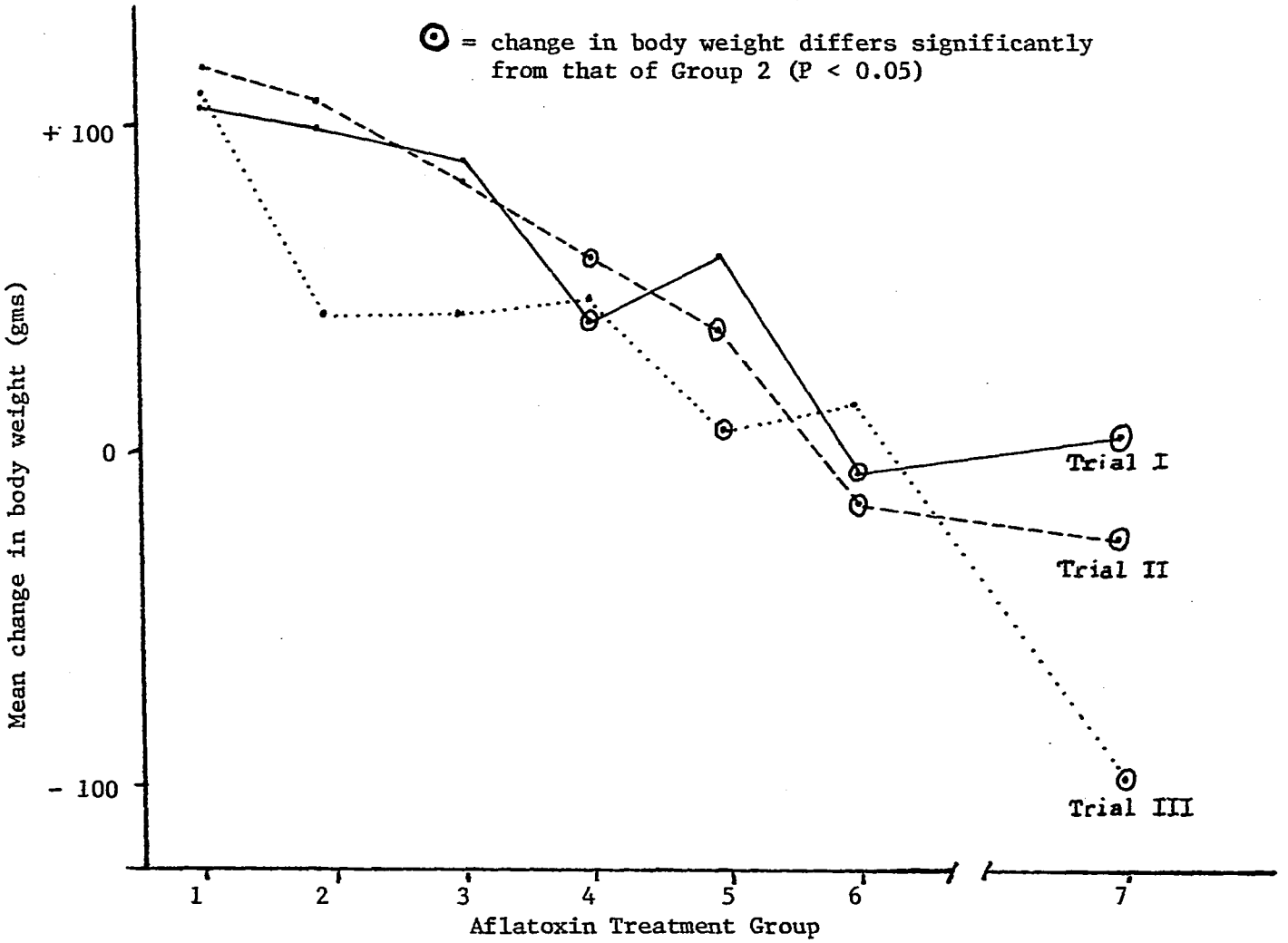

Figure 1 Mean change in body weight from day 0 to day 24 in aflatoxin-treated guinea pigs

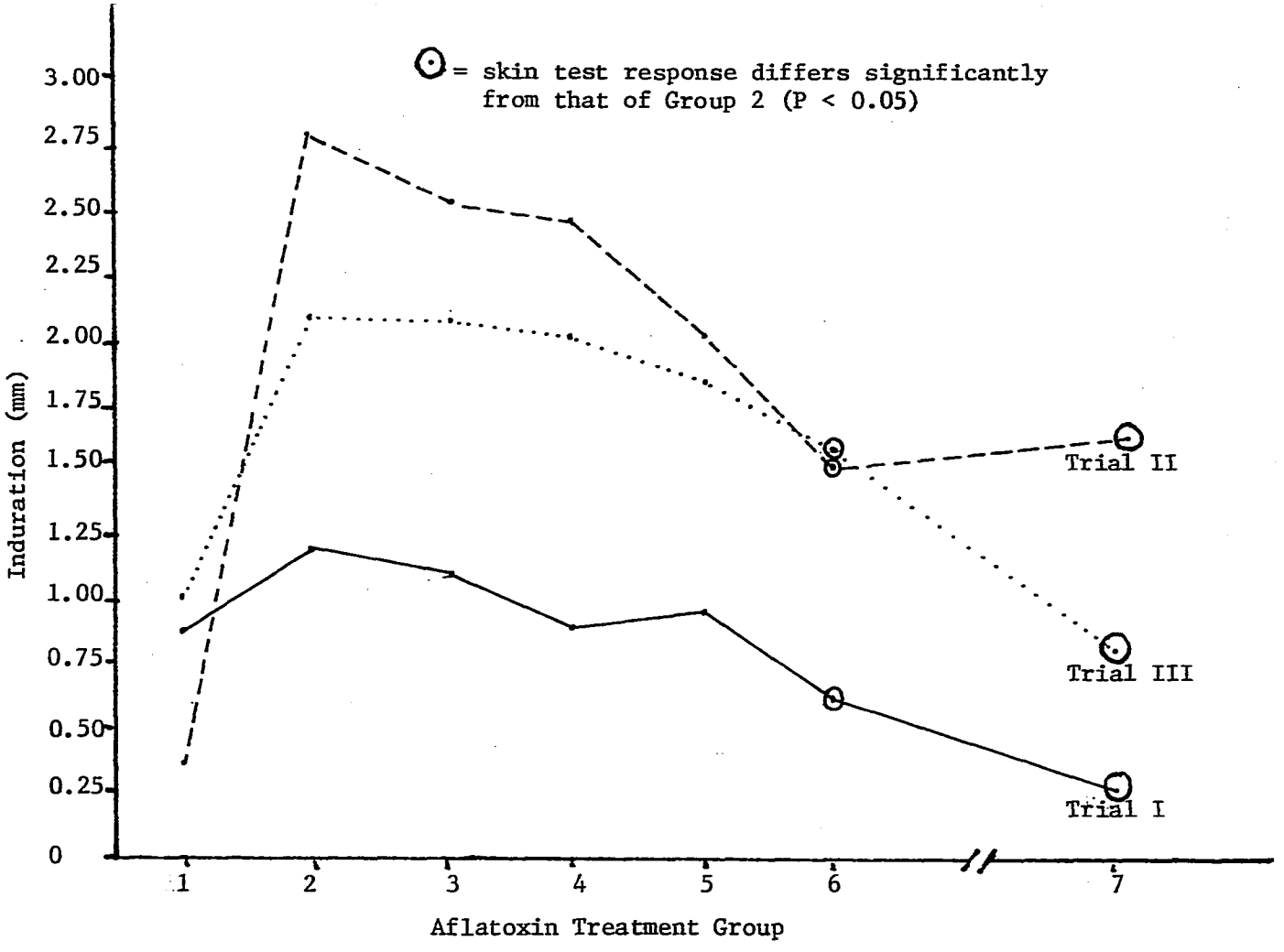

Figure 2 Mean skin test response of aflatoxin-treated guinea pigs

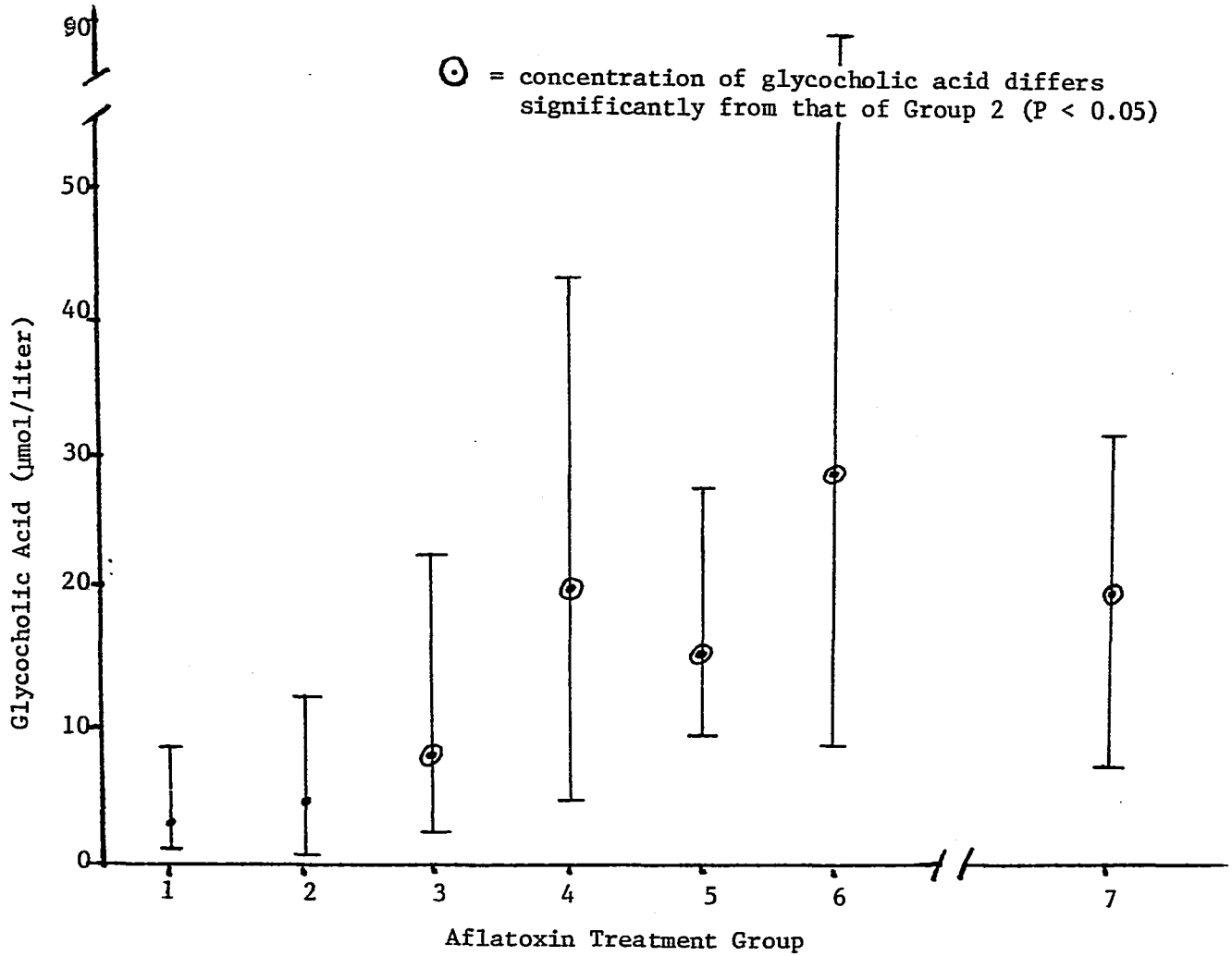

Figure 3 Serum concentrations of glycocholic acid in aflatoxin-treated guinea pigs

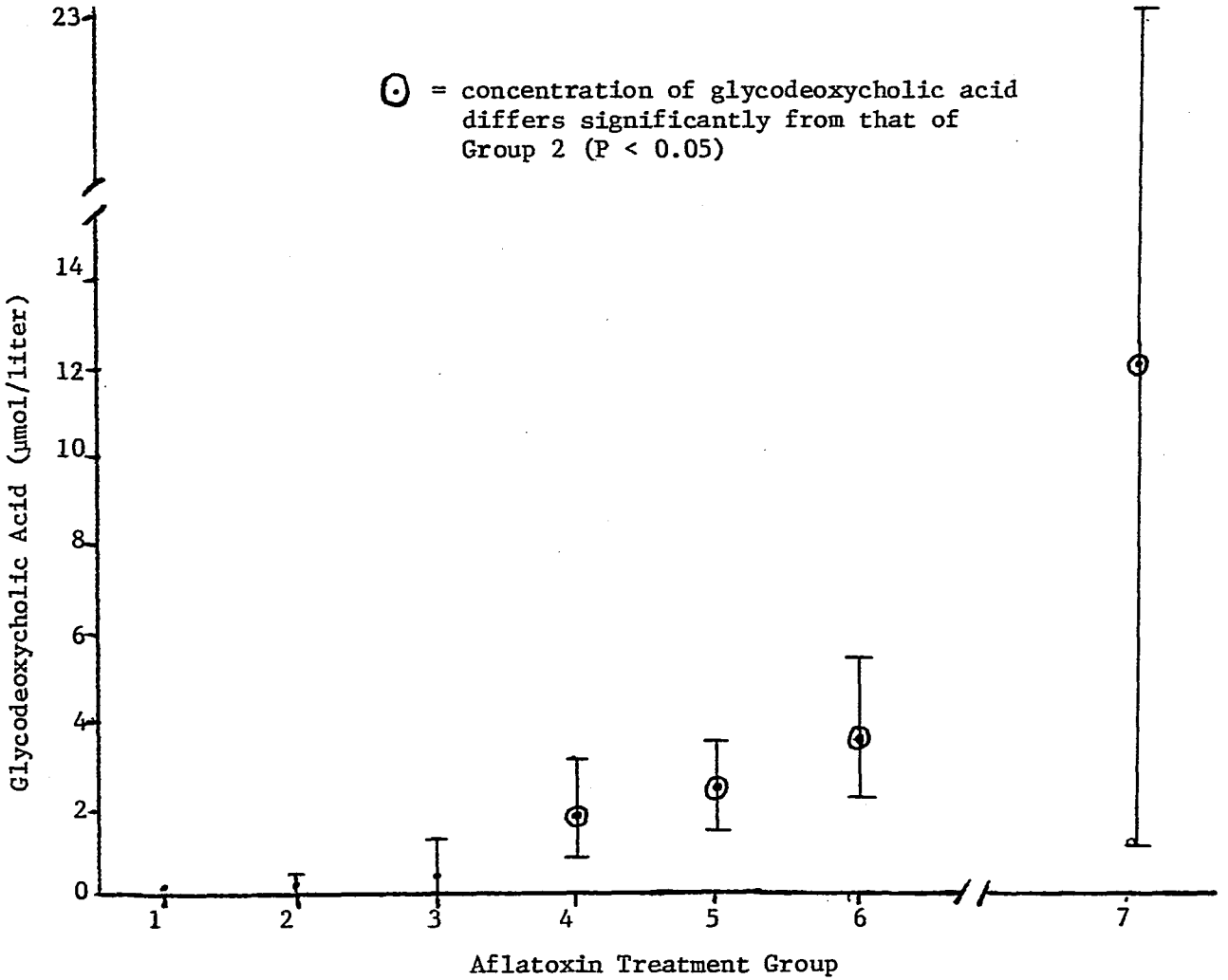

Figure 4 Mean and range of serum concentration of glycodeoxycholic acid in aflatoxin-treated guinea pigs

Cytomegaly and sinusoidal dilation characterized the histologic changes in liver tissue from guinea pigs in dose groups 6 and 7. Such changes typify chronic sublethal ingestion of aflatoxin in guinea pigs.[12] Hepatopathic changes were observed in tissues from some guinea pigs in lower dose groups but other animals in these groups had normal liver histology. As seen in Fig 3, serum glycocholic acid was elevated in all aflatoxin-treated guinea pigs (groups 3, 4, 5, 6, and 7 ($P < 0.05$)). The level of glycodeoxycholic acid increased with dose, becoming elevated at dose level 4 (Fig 4). Among guinea pigs given the same dose of aflatoxin, wide variations in serum concentrations of bile acids were observed, ranging from near normal levels to greater than ten times the level detected in control guinea pigs.

In the three trials, there was no mortality in groups 1, 2, 4, or 5. A subcutaneous abscess in the cervical region was found during postmortem examination of a single animal in group 3 (Trial II) which died on day 15 of aflatoxin treatment. The liver from this animal was normal grossly and microscopically. Two out of 30 animals in group 6 (1 from Trial II, 1 from Trial III) died during treatment. Both evidenced jaundiced skin, extensive hair loss and were below pretreatment weight. Histologic examination of sectioned liver tissue from these animals showed extensive hepatocytomegaly and sinusoidal dilation. Twelve out of thirty animals in group 7 (4 from Trial I, 2 from Trial II, 6 from Trial III) died during treatment. They too registered a significant weight loss and evidenced hepatopathic changes consistent with chronic sublethal ingestion of aflatoxin.

DISCUSSION

The results substantiate earlier studies in which reduced weight gains were observed at 0.030 mg/kg/day B_1 eq.[13] In trial I, adequate sensitization was not demonstrated by skin testing (compare groups 1 and 2, Fig 2). In trials II and III, adequate sensitization was achieved as demonstrated by the maximal skin test response of untreated sensitized guinea pigs in group 2 versus unsensitized control animals in group 1 (Fig 2). Despite the low level of sensitization in trial I, the dose-related effect of aflatoxin on skin test response was evident in all trials. However, disparity between trials necessitated separate statistical analysis for each trial.

Effects of aflatoxin on skin test responsiveness became statistically significant at 0.040 mg/kg/day B_1 eq. Reduced skin test responses were observed in some individuals which received as low as 0.020 mg/kg/day B_1 eq but were masked in the group mean by wide variations in individual susceptibility to aflatoxin. At the highest dose used in these experiments (0.070 mg/kg/day B_1 eq), mortality removed the highly susceptible animals from the group. The mean skin test response for the group would probably have been considerably lower had these animals lived to be tested. Individual variation in susceptibility to aflatoxin has been noted for a variety of species,[14,15] including guinea pigs,[13] and is probably related to individual differences in metabolism.

Determination of serum bile acids was a more sensitive indicator of liver changes than was histologic examination of liver tissue. Elevated concentrations of glycocholic acid were detectable at low doses of afla-

toxin. However, the concentration of glycodeoxycholic acid, which increased in a dose-related manner, may be a more reliable indicator of the amount of aflatoxin consumed.

The suppressive effect of aflatoxin on delayed-type hypersensitivity was first observed in guinea pigs at a level of approximately 0.070 mg/kg/day B_1 eq by Pier et al.[1] In the present study, aflatoxin-induced immunosuppression was demonstrated to occur at lower levels of aflatoxin ingestion (0.040 mg/kg/day B_1 eq) and was related to concomitant changes in weight gain and serum bile acid levels.

Inhibition of protein synthesis caused suppression of delayed hypersensitivity *in vitro* in guinea pigs.[16] The mechanism(s) by which aflatoxin causes immunosuppression may be referable to an inhibition of protein synthesis.[14] Reduced skin test responses caused by aflatoxin may reflect functional or quantitative changes in lymphocyte or macrophage populations, impaired interactions between these populations, activation of suppressor lymphocytes, or any combination of these events.

SUMMARY

Guinea pigs received daily oral doses of aflatoxin ranging from 0.010 to 0.070 mg/kg/day aflatoxin B_1 equivalents (B_1 eq) for 21 days. Delayed type hypersensitivity and weight gain were evaluated at the end of aflatoxin treatment. Aflatoxin affected skin test responsiveness and weight gain in a dose-related manner. A reduction in delayed type hypersensitivity, as measured by skin test responsiveness to a Nocardia asteroides sensitin, was observed in the groups of guinea pigs which received at least 0.040 mg/kg/day B_1 eq. Groups which received at least 0.020 mg/kg/day B_1 eq demonstrated reduced weight gains. Serum bile acid levels, used to evaluate liver damage in aflatoxin treated guinea pigs, revealed elevated concentrations of glycocholic and glycodeoxycholic acid in guinea pigs which received at least 0.010 and 0.020 mg/kg/day B_1 eq, respectively.

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SECTION II. PASSIVE TRANSFER OF DELAYED TYPE HYPERSENSITIVITY IN AFLATOXIN-TREATED GUINEA PIGS

INTRODUCTION

Aflatoxin suppresses delayed type hypersensitivity,[1,2] lymphokine activity,[1] and lymphoblastogenesis[1] in guinea pigs in the face of normal antibody production.[3] Cell-mediated immune responses appear to be preferentially sensitive to aflatoxin, suggesting a role for T lymphocytes in the observed immunosuppression. No change in absolute numbers of total lymphocytes or T or B lymphocytes in peripheral blood was observed in guinea pigs[4] at levels of aflatoxin ingestion which caused reduced weight gain, increased serum concentrations of bile acids, and depressed skin test responsiveness in similar studies.[2]

In the present experiment, passive transfer of delayed type hypersensitivity by sensitized T lymphocytes was chosen as a means for assaying the functional activity of T lymphocytes from aflatoxin-treated guinea pigs because: (1) it permitted <u>in vivo</u> analysis of cell-mediated immune processes and (2) the technique in guinea pigs has been well described.

MATERIALS AND METHODS

Animals--Female guinea pigs (400-450 g) from a closed colony at the National Animal Disease Center (Ames, IA) were randomly assigned to 'donor' or 'recipient' groups. Two donor guinea pigs were allotted per recipient. All guinea pigs received water and pelleted guinea pig ration (Teklad, Monmouth, IL) ad libitum.

Aflatoxin--A partially purified preparation of aflatoxins (PPA) was used that contained 47% aflatoxin B_1 equivalents (B_1 eq).[2] PPA was dissolved in chloroform and dispensed into gelatin capsules (Size #5, Lilly Pharmaceutical, Indianapolis, IN). Animals in appropriate groups (Fig 1) were given a daily dose of 0.050 mg/kg B_1 eq per os for 24 days or served as nontreated controls.

Passive transfer of delayed type hypersensitivity--The method of Bloom and Chase[5] for passive transfer of delayed type hypersensitivity to tuberculin was used with some modifications. All donor groups were sensitized to Nocardia asterodies as previously described.[6] On day 24, donor guinea pigs were given a single intraperitoneal injection of 25 ml Bayol-55 (Humble Oil Co., Houston, TX) to induce a peritoneal exudate containing primarily lymphocytes and macrophages. On day 26, these animals were sacrificed and peritoneal exudate cells (PEC) were harvested by exhaustive peritoneal lavage. PEC collected from two donor guinea pigs contained enough cells to inoculate a single recipient guinea pig. Pooled PEC from aflatoxin-treated or nontreated guinea pigs were used to inoculate recipient guinea pigs, each of which received an intraperitoneal injection of 3×10^8 cells. Stained (Volu-Sol Stat Strain, Volu-Sol Medical Industries,

Las Vegas, NV) smears of each PEC pool were prepared for differential leukocyte count. Cell viability was determined by exclusion of 0.05% Trypan blue dye. Unsensitized recipient guinea pigs were skin tested by intradermal injection (0.1 ml) of homologous N asteroides culture filtrate sensitin on day 27 (one day following transfer of sensitized cells). Induration, measured with calipers (in millimeters), was recorded at 24 hours:

Induration = skin thickness at test site - skin thickness at medium-injected control site

Control guinea pigs, which were sensitized and/or aflatoxin-treated but did not receive transferred cells, were skin-tested at this time as well.

Statistical analysis--Three trials of the experimental design were conducted. Cumulative results were analyzed using Dunnett's test for analysis of variance.[7]

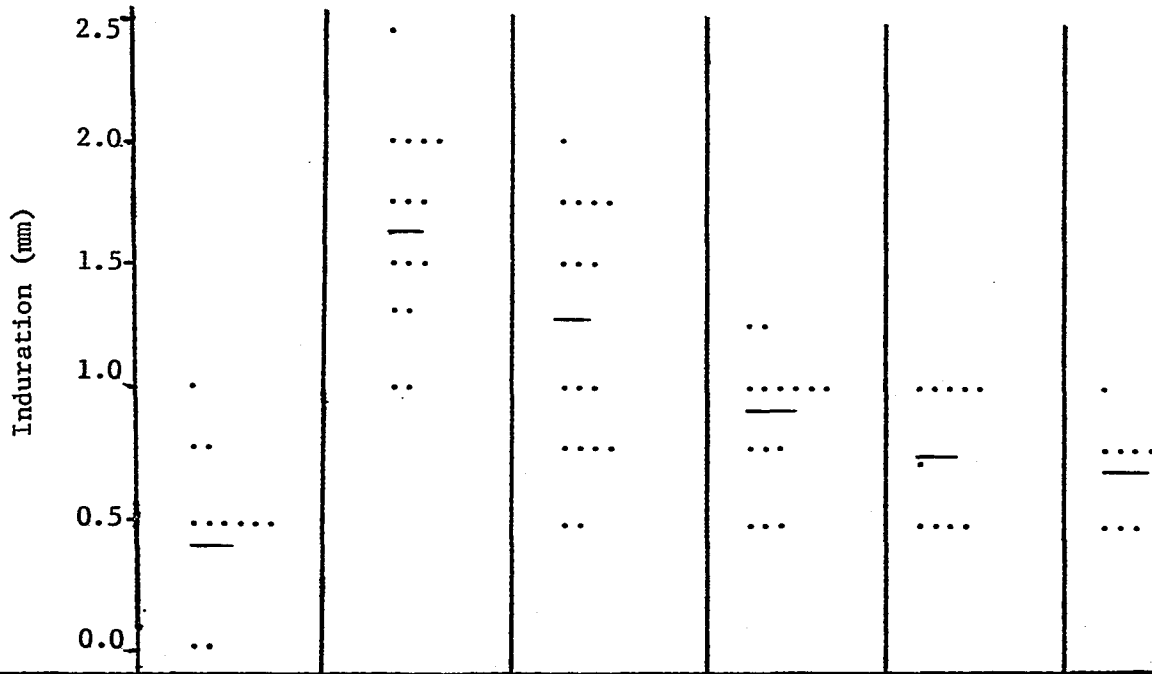
RESULTS

The differential leukocyte count and viability of donor PEC were similar for PEC pools from nontreated and aflatoxin-treated guinea pigs. This permitted comparisons between the skin test responses of recipients inoculated with PEC from nontreated and aflatoxin-treated guinea pigs.

Individual skin test response, mean skin test response for the group, and mortality are shown in Fig 1. Groups 1, 2, and 3 were control groups. The mean skin test responses of sensitized, nontreated guinea pigs in group 2 (induration = 1.65 mm) and sensitized, aflatoxin-treated guinea pigs in group 3 (1.19 mm) were both significantly larger ($P < 0.05$) than the mean skin test response of nonsensitized, nontreated guinea pigs in group 1 (0.43 mm). The skin test response of aflatoxin-treated guinea pigs in group 3 was smaller ($P < 0.05$) than that of nontreated guinea pigs in group 2.

Groups 4, 5, and 6 were nontreated or aflatoxin-treated, nonsensitized recipient guinea pigs into which were transferred PEC from nontreated or aflatoxin-treated, sensitized guinea pigs. In group 4, nontreated recipients received PEC from nontreated guinea pigs; the skin test response of group 4 represented the maximum skin test response of recipient guinea pigs. The mean skin test response of group 4 (0.88 mm) was significantly larger ($P < 0.05$) than that of nonsensitized controls (group 1), establishing the specificity of the passive transfer reaction.

The mean skin test responses of groups 5 and 6 were numerically lower than that of group 4. The distribution of skin test responses for group 6 was different when compared to the responses of guinea pigs in group 4



	Group	1	2	3	4	5	6
	Mortality	0/15	0/15	3/20	0/14	0/11	6/14
PEC†	Sensitized with N asteroides	NO PEC transferred			+*	+	+
	Aflatoxin				-	+	-
Recipient	Sensitized with N asteroides	-	+	-	-	-	-
	Aflatoxin	-	-	+	-	-	+

*+ = received indicated treatment.

†PEC = peritoneal exudate cells from sensitized, nontreated or aflatoxin-treated guinea pigs.

Figure 1 Effect of aflatoxin on passive transfer of delayed type hypersensitivity

(Fig 1); the response of 7 out of 8 guinea pigs in group 6 was below the mean skin test response of group 4. In this study there were no statistically significant differences in the skin test responses of nontreated recipients which received PEC from aflatoxin-treated guinea pigs (group 5) or aflatoxin-treated recipients which received PEC from nontreated guinea pigs (group 6) when compared with the skin test response of nontreated recipients which received PEC from nontreated guinea pigs (controls, group 4).

Individual variation in susceptibility to aflatoxin[8] minimized the usefulness of statistical analysis[6] in the present experiment. No mortality was observed for any nontreated guinea pigs. Greater than 10% mortality (3/20) was noted in group 3 which consisted of sensitized, aflatoxin-treated guinea pigs that did not receive any PEC. Group 6 registered greater than 40% mortality. Animals in this group were aflatoxin-treated guinea pigs which received PEC.

In three trials, all deaths among animals in group 6 occurred following inoculation with PEC. No deaths were recorded for animals in group 4 which were inoculated with PEC from the same pool. Had the highly susceptible animals in group 6 lived to be skin tested, the overall group response would have been considerably lower. As it was, only the most aflatoxin-resistant (and probably more immunologically responsive) guinea pigs in group 6 lived to be tested.

DISCUSSION

The results presented herein confirm previous demonstrations of diminished skin test responsiveness[1] associated with aflatoxin ingestion and provide some insight into the mechanism underlying the observed immunosuppression. It appears that aflatoxin interferes with delayed type hypersensitivity reactions at both the affector and effector levels. Since the cellular composition and viability of PEC from nontreated and aflatoxin-treated guinea pigs was comparable, the diminished skin test response may have resulted, in group 5, from some functional alteration in the transferred PEC. Altered function may result from altered activity (lymphokine production)[1] or cell surface characteristics of individual participating lymphocytes or it may reflect critical shifts, undetectable by a differential leukocyte count, in subpopulations comprising the T lymphocyte population.[4]

The diminished skin test responses of animals in group 6 may reflect, in part, an altered physiological state resulting from aflatoxin's hepatotoxic action.[9] Since the guinea pigs in group 6 received fully sensitized T lymphocytes contained in PEC from nontreated guinea pigs, antigen processing was not a factor. Aflatoxin, which impaired phagocytic activity of rabbit alveolar macrophages,[10] may have altered the ability of macrophages to be recruited to a skin test site in response to a lymphokine signal from a sensitized T lymphocyte. Although the T lymphocytes contained in PEC transferred to recipients in group 6 were from nontreated donor guinea pigs, they may have been introduced into a suppressive environment when infected into the aflatoxin-treated recipient guinea pig.

SUMMARY

A passive transfer model was used to evaluate the relative contributions of sensitized T lymphocytes and macrophages from aflatoxin-treated guinea pigs to the resultant skin-test reaction. The mean caliper measurement of induration (mm) at the skin test site was 0.88 mm for nontreated recipient guinea pigs which received peritoneal exudate cells (PEC) from nontreated, sensitized guinea pigs. Nontreated recipients inoculated with PEC from aflatoxin-treated, sensitized guinea pigs had a mean skin test response of 0.80 mm. Aflatoxin-treated guinea pigs receiving PEC from nontreated, sensitized guinea pigs had a mean skin test response of 0.72 mm. These differences were not statistically significant, possibly due to individual variation in susceptibility to aflatoxin.

Aflatoxin-treated recipient animals had 40% mortality after inoculation with PEC; nontreated recipients given PEC from the same preparation had 0% mortality. Aflatoxin appeared to cause specific immunosuppression by altering some functional capacity of T lymphocytes and macrophages.

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SECTION III. BACTERIA AND YEASTS AS POTENTIAL LYMPHOCYTE MARKERS IN GUINEA PIGS

INTRODUCTION

Some lymphocytes spontaneously bind bacteria to their surfaces. This property was used by Teodorescu and co-workers to develop a bacterial binding assay for identifying human[1] and murine[2] lymphocyte populations and subpopulations. Lymphocyte subpopulations isolated by this method had functional analogy to lymphocytes traditionally identified by mitogen responsiveness, allogeneic reactivity and cytotoxic activity.[3,4] Bacterial markers were used to enumerate normal lymphocyte populations and subpopulations[1] and to detect seasonal variations therein.[5] The bacterial binding assay also proved useful in the diagnosis of such diseases as lymphocytic leukemia.[6]

In guinea pigs, B and T lymphocytes currently are distinguished, respectively, by the presence of surface immunoglobulin (sIg) or the ability to form rosettes with heterologous erythrocytes. These methods require purified lymphocyte preparations and immediate interpretation using phase contrast microscopy. The bacterial binding assay developed by Teodorescu and co-workers offers several advantages: 1. There is no depletion of subpopulations because whole blood is used; 2. the low background of bacteria around other cell types and nonbinding lymphocytes facilitates identification of rosettes; 3. other cell types, notably monocytes, may show rosetting but are easily distinguished from lymphocytes in stained blood smears; 4. the rosettes which form during a six minute centrifugation are stable through three washes, vortexing, and smear procedure; 5. identification of rosettes in stained smears precludes the necessity for

phase-contrast microscopy and eliminates the need for immediate reading of results; 6. reagents are inexpensive, uniform and stable for at least six months.[7]

The objective of this study was to determine if bacteria and yeasts could be used to identify lymphocyte populations in guinea pig peripheral blood and lymphoid tissues.

MATERIALS AND METHODS

Animals--Female guinea pigs (400-450 g) from a closed colony at the National Animal Disease Center (Ames, IA) were used in all experiments. They received pelleted guinea pig ration (Teklad, Monmouth, IL) and water ad libitum.

Preparation of bacteria and yeasts--The genera of bacteria and yeasts used in these experiments are listed in Table 1. Bacteria were grown for 48 hours at 38 C in antibiotic medium (Difco Laboratories, Detroit, MI). Yeasts were grown for 24 hours at 25 C in potato-dextrose broth (Difco Laboratories, Detroit, MI). Organisms were harvested as previously described.[7] Cultures were incubated with 0.05% merthiolate (final concentration) for 1 hour at 37 C, then centrifuged at 8,000 x g for 5 min and washed 3x in phosphate buffered saline (PBS, pH 7.4). Formalin (final concentration 10%) was added to the washed cultures which were then incubated for 24 hours at 4 C, harvested by centrifugation and washed 3x in PBS as above. Killed organisms were resuspended in PBS without sodium azide to yield an approximate concentration of 10^9 cells/ml.

Bacterial binding assay--Five to ten ml of blood was obtained by cardiac punture from guinea pigs anaesthetized with carbon dioxide. The blood was immediately transferred to tubes containing 143 USP units of sodium heparin. The bacterial binding assay was performed as previously described.[7] Briefly, whole blood or lymphocyte suspensions were washed three times in Eagle's minimum essential medium (Gibco, Grand Island, NY) containing 6% bovine serum albumin (Fraction V) (MEM-BSA) with or without

0.02% sodium azide. Samples were then mixed and centrifuged with formaldehyde-fixed bacteria or yeasts and washed as above to remove unbound bacteria. Bacterial adherence was determined by microscopic examination of 300 lymphocytes in stained smears (Volu-Sol Stat Stain, Volu-Sol Medical Industries, Las Vegas, NV).

Lymphocyte preparations--Peripheral blood lymphocytes (PBL) were separated from heparinized blood by Ficoll-Hypaque (sp. gr. 1.080) density gradient centrifugation.[8] To obtain suspensions of lymphocytes from lymphoid organs, lymph node, thymus, or spleen was aseptically excised and placed in Hank's balanced salt solution containing phenol red. Tissues were gently minced with scissors and expressed through a fine mesh wire screen filter (Millipore, 60 mesh, Bedford, MA) to separate cells from fibrous connective tissue stroma. Ficoll-Hypaque purified mononuclear cells and lymphocyte suspensions were washed three times in MEM-BSA. Cell suspensions were counted in a Neubauer Hemacytometer after dilution with 20 µl Unopettes (Becton-Dickinson, Rutherford, NJ) and concentrations were adjusted to 1×10^7 cells/ml. Lymphocyte preparations used in these studies demonstrated >90% viability by trypan blue (0.5%) exclusion.

A preparation of mononuclear cells enriched in T-lymphocytes was obtained by passage of Ficoll-Hypaque purified PBL (pooled from 3 guinea pigs) over adherence columns containing nylon wool (Fenwal Laboratories, Division of Travenol Laboratories, Morton Grove, IL).[9] After 45 min of incubation at 37 C, nonadherent cells were eluted by washing with prewarmed media (MEM containing 1% BSA). These 'first-wash' cells were applied to a

second column, eluted in a similar manner, and cell concentrations were adjusted to 1 x 10^7 cells/ml. 'Second-wash' cells proved to be >95% T cells as determined by indirect immunofluorescence with an anti-T cell monoclonal antibody directed against guinea pig peripheral blood T lymphocytes (Dr. Ethan Shevach, NIH). These 'second-wash' cells were designated T-enriched lymphocytes.

Scanning electron microscopy--Air-dried stained blood smears were mounted on aluminum stubs which were then coated with silver paint, lightly gold shadowed and examined by SEM.

RESULTS

Studies indicated that the presence of as little as 0.02% sodium azide in the washing medium (MEM-BSA) markedly inhibited the ability of guinea pig PBL to form rosettes with several strains of bacteria (Table 1). Sodium azide was omitted from the washing medium in all experiments with guinea PBL, or guinea pig lymphocytes derived from lymphoid tissues.

None of ten yeasts examined were bound by guinea pig PBL. Fourteen of fifty-three bacterial strains screened were observed to bind >5% of peripheral blood lymphocytes (Table 2). An approximate measure of binding affinity was obtained by counting the number of bound bacteria per lymphocyte. For example, both Yersinia enterocolitica and Y pseudotuberculosis were bound by guinea pig PBL. Greater than 20 Y enterocolitica organisms surrounded a single lymphocyte; whereas, only 3-5 Y pseudotuberculosis organisms were observed around a lymphocyte in a preparation from the same animal. Only those strains of bacteria whose morphology permitted easy identification of rosettes and which consistently demonstrated a high binding affinity were chosen for further study. These bacteria and the relative percentage of guinea pig PBL labelled by each are shown in Table 3. A typical rosette is shown in Fig 1. Examination of rosettes using scanning electron microscopy revealed the close association of bacteria with the lymphocyte cytoplasmic border (Fig 2).

A comparison of the binding of bacteria by PBL and lymphocytes derived from lymphoid tissues is shown as a mean for four guinea pigs in Fig 3. The percentage of lymphocytes that bound a given species of bacterium

Table 1 Effect of sodium azide on binding of bacteria by guinea pig peripheral blood lymphocytes

	Percent PBL labelled	
	Sodium azide concentration	
Bacteria	0.02%	0.000%
Salmonella schottmulleri	9	48
Brucella melitensis	NB*	11
Arizona hinshawii	NB	12
Bacillus globigii	6	11

*NB = nonbinding (mean <5%).

Table 2 Genera of bacteria and yeasts screened in bacterial binding assay

General classification		Genus†	No. strains screened	Strains which bind* Guinea pig PBL
Gram negative	Facultative anaerobes	Arizona	1	1
		Citrobacter	1	1
		Enterobacter	1	0
		Escherichia	8	1
		Klebsiella	2	1
		Salmonella	4	2
		Actinobacillus	1	0
		Pasteurella	3	0
		Yersinia	2	2
	Aerobic rods and cocci	Alcaligenes	1	0
		Brucella	6	1
		Bordetella	1	0
		Pseudomonas	2	0
Gram positive	Asporogenous rod shaped	Erysipelothrix	2	0
		Listeria	1	0
	Endospore forming rods	Bacillus	2	1

	Cocci	Micrococcus	2	0
		Staphylococcus	3	0
		Streptococcus	8	0
Actinomycetes		Corynebacterium	3	0
Yeasts		Candidia	8	0
		Hansenula	1	0
		Torulopsis	1	0

*Positive = >5% PBL bound.

†Isolates were obtained from Dr. Marius Teodorescu, University of Illinois Medical Center; Dept. of Veterinary Microbiology, Iowa State University; or National Animal Disease Center, Ames, Iowa.

Table 3 Percentage of guinea pig peripheral blood lymphocytes labelled with different strains of bacteria

Bacteria	Percent PBL labelled		
		Range	
	Mean*	Minimum	Maximum
Salmonella schottmulleri	48	34	65
Brucella melitensis	11	3	20
Yersinia enterocolitica	12	7	19
Bacillus globigii	11	8	16
Arizona hinshawii	12	9	14
Escherichia coli (hemolytic)	NB†	0	7
Escherichia coli (smooth)	NB	0	3

*n = 12 guinea pigs.

† NB = mean was <5%.

Table 4 Percentage of untreated and T-enriched guinea pig peripheral blood lymphocytes labelled with different strains of bacteria

Bacteria	Untreated	Enriched
Salmonella schottmulleri	51	84
Brucella melitensis	15	4
Yersinia enterocolitica	18	27

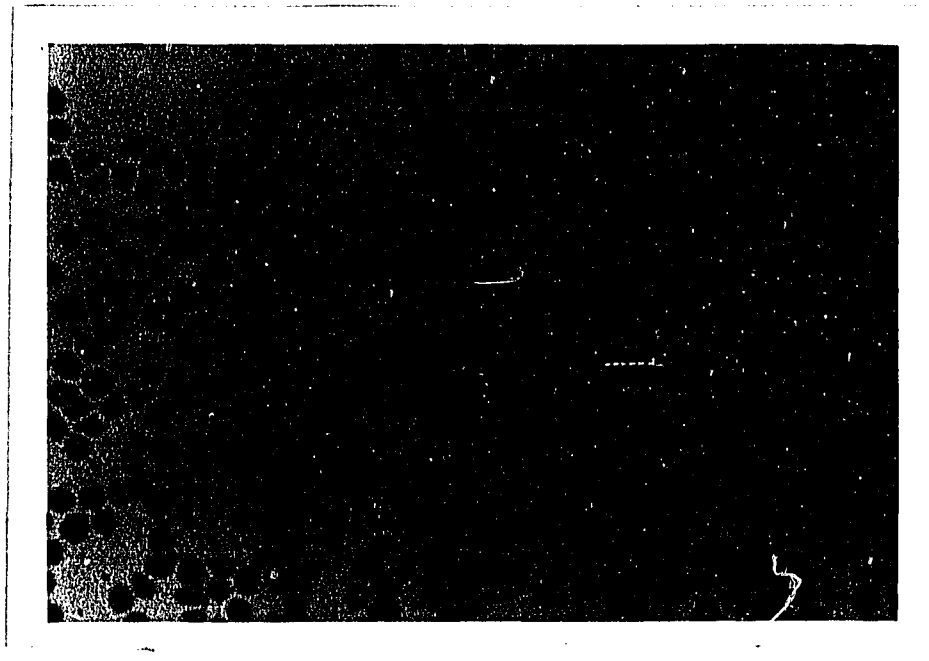

Figure 1 A typical rosette. Note the numerous bacteria (Salmonella schottmulleri) surrounding a guinea pig lymphocyte, the adjacent nonbinding lymphocyte, and low background of bacteria in the stained blood smear (750X)

Figure 2 Scanning electron micrograph showing a guinea pig lymphocyte surrounded by Salmonella schottmulleri organisms (3700X)

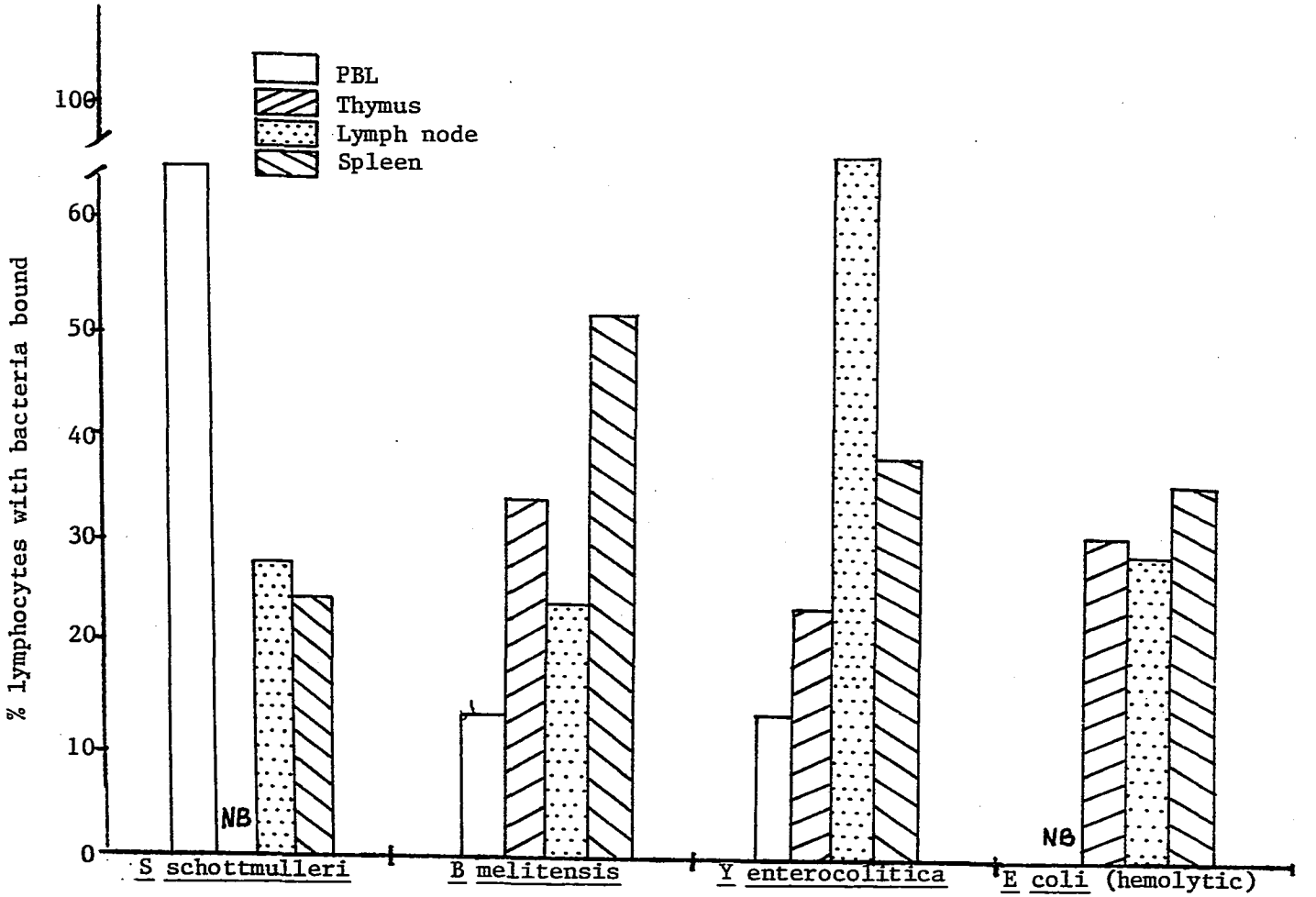

Figure 3 Binding of bacteria to guinea pig lymphocytes from peripheral blood and lymphoid tissues

varied in different tissues. Salmonella schottmulleri was bound by a high percentage of PBL (49%), was not bound by guinea pig thymocytes and was bound, respectively, by 18% and 22% of splenic lymph node-derived lymphocytes. Brucella melitensis, bound by 10% of PBL, was bound by 26%, 17%, and 38% of thymocytes, lymph node derived lymphocytes, and splenic lymphocytes. Yersinia enterocolitica was bound by different percentages of lymphocytes derived from lymphoid tissues (18% thymocytes, 51% lymph node derived and 30% splenic lymphocytes) compared to PBL (11%). A hemolytic strain of E coli was not bound by PBL, but was bound by the same number of lymphocytes (23-28%) derived from thymus, lymph node, or spleen.

The relative percent of bacteria bound by T-enriched preparations of PBL and untreated PBL is compared in Table 4. The data are from pooled PBL from three guinea pigs. Relative to PBL, T-enriched lymphocytes bound more S schottmulleri and Y enterocolitica. Eighty-four percent of the T-enriched lymphocytes bound S schottmulleri as compared to 51% of the untreated PBL. The percentage of T-enriched lymphocytes binding Y enterocolitica (27%) was 1.5 times as large as the percentage of PBL (18%) binding this bacterium. However, the percent of T-enriched lymphocytes which bound S schottmulleri was 84% compared to 27% Y enterocolitica-positive T-enriched lymphocytes. Binding of B melitensis was markedly decreased in the T-enriched preparation of lymphocytes (4%) compared to PBL (15%).

DISCUSSION

Binding of bacteria by lymphocytes is not dependent on previous exposure to the organism as lymphocytes from gnotobiotic mice bind bacteria to the same extent as lymphocytes from normal mice.[10] However, pathogenicity of the bacterium may play a role in determining the ability of lymphocytes to form rosettes with a bacterial species and may affect the quality of rosettes which form. For example, nonpathogenic strains of Brucella were bound less than pathogenic strains.[11]

The proposed mechanism of binding involves interactions between lectin-like molecules on the lymphocyte and carbohydrate moieties, in particular D-mannose, on the bacterium.[12] This challenges numerous reports[13] in which adhesion of microorganisms to mammalian cell surfaces occurs via lectins on bacteria which react with carbohydrates on lymphocytes. In the present study, none of the yeasts was observed to bind to PBL despite "moderate" levels of mannose in the cell wall.[14]

Low concentrations of sodium azide (0.02%) markedly inhibited the ability of guinea pig PBL to form rosettes with bacteria, in particular S schottmulleri. The ability of guinea pig T lymphocytes to form rosettes with heterologous red blood cells is also inhibited in the presence of sodium azide,[15] suggesting a common basis for the observed inhibition.

The binding of bacteria by lymphocytes derived from guinea pig thymus, lymph node, and spleen was examined in an effort to identify the lymphocyte populations which bound various bacteria. The results were unexpected in view of the identity of human and murine peripheral blood lymphocytes which bound these bacteria.[10] These results cannot be explained at this time but

may reflect maturational differences between lymphocytes in peripheral blood and those in lymphoid tissues.

The binding of bacteria by a T-enriched preparation of lymphocytes obtained by passing Ficoll-Hypaque purified mononuclear cells over nylon wool adherence columns was examined. Substantial differences in reactivity between T-enriched PBL and untreated PBL were observed (Table 4) due to the relative increase in T lymphocytes and relative decrease in B lymphocytes. The results indicated that S schottmulleri, identified as a marker for human B lymphocytes and most T lymphocytes,[16] was bound by a large proportion of guinea pig T lymphocytes. Enumeration of T lymphocyte populations in guinea pigs using immunofluorescence and bacterial markers showed a high correlation between the percentage of PBL identified by immunofluorescence as T lymphocytes (44.7%) and those labelled by S schottmulleri (40.5%).[17] These values, and the values presented herein (48%), are in accord with values obtained using heterologous erythrocyte rosette formation to identify T lymphocytes (45%).[15] Yersinia enterocolitica appeared to label a subpopulation of T lymphocytes. Brucella melitensis appeared to be a B lymphocyte marker in guinea pigs as it is in humans and mice. The number of PBL which bound B melitensis was within the range of numbers of B lymphocytes identified in peripheral blood in other studies.[18]

Several strains of bacteria were identified which may be useful markers for studying lymphocyte populations in guinea pigs. Salmonella schottmulleri and Y enterocolitica was bound by some portion of T lymphocytes and B melitensis was bound by B lymphocytes.

SUMMARY

Fifty-three bacteria and yeasts were screened to identify strains which were bound consistently by peripheral blood lymphocytes (PBL) of guinea pigs. None of ten yeasts examined was bound by guinea pig PBL. Ten of fifty-three bacterial strains were bound by >5% of PBL. Potential lymphocyte markers from these bacteria were chosen for further study. Sodium azide inhibited the binding of bacteria by PBL.

T-enriched lymphocyte preparations indicate that Salmonella schott-mulleri was bound by most T lymphocytes; Yersinia enterocolitica was bound by a subpopulation of lymphocytes therein; and Brucella melitensis was bound by B lymphocytes. Patterns of binding of bacteria by lymphocytes from various lymphoid tissues diverged from patterns obtained using T-enriched preparations of PBL. The data indicated that maturational events which occur in thymus, lymph node, and spleen alter the cell surface characteristics of lymphocytes by which lymphocytes bind bacteria.

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SECTION IV. T AND B LYMPHOCYTE POPULATIONS IN AFLATOXIN-TREATED GUINEA PIGS

INTRODUCTION

Aflatoxin has been shown to suppress cell-mediated immune processes in guinea pigs.[1] Daily oral administration of 0.040 mg aflatoxin B_1 equivalents (B_1 eq) per kilogram body weight during a three week period was sufficient to reduce skin test responsiveness in guinea pigs.[2] In similar experiments, antibody production was not affected by aflatoxin at even higher dose levels (0.060 mg/kg/day B_1 eq).[3] Immunologic consequences of aflatoxin ingestion were the subject of a recent review.[4]

Aflatoxin's immunosuppressive effect on cell-mediated immune processes has been demonstrated for a variety of domestic and laboratory animal species utilizing *in vivo* and *in vitro* assays.[5] Aflatoxin-induced changes include thymic involution,[6] reduced delayed type hypersensitivity,[1,2] reduced lymphoblastogenesis,[7,8] and diminished graft vs host response.[9] The immunosuppressive effect may reflect a functional or quantitative change in lymphocyte populations.

A precise analysis of aflatoxin's mode of action on the guinea pig cellular immune system has not been feasible due to limited methodology. Recently, we identified bacterial strains which were useful in identifying guinea pig T and B lymphocyte populations in peripheral blood.[10] In the present study, we compared the numbers of T and B lymphocytes in peripheral blood from aflatoxin-treated and normal guinea pigs using immunofluorescence and bacterial markers to identify lymphocyte populations. *Salmonella schottmulleri* and *Yersinia enterocolitica*, identified as markers for some

guinea pig T lymphocytes on the basis of binding by lymphocytes in T-enriched lymphocyte preparations,[10] were used to detect changes in lymphocyte subsets in aflatoxin-treated guinea pigs.

MATERIALS AND METHODS

Animals--Female guinea pigs (400-450 g) from a closed colony at the National Animal Disease Center (Ames, IA) were used. Thirty guinea pigs were randomly distributed into five experimental groups (n = 6) and received water and pelleted guinea pig ration (Teklad, Monmouth, IL) *ad libitum*.

Aflatoxin--A partially purified preparation of aflatoxins (PPA) containing 47% B_1 equivalents was used as previously described.[11,12] The PPA was dissolved in chloroform and dispensed into gelatin capsules (Size #5, Lilly Pharmaceutical, Indianapolis, IN). Animals in groups 1 through 5, respectively, received 0.000, 0.020, 0.030, 0.040, or 0.060 mg/kg/day B_1 eq *per os* for 3 weeks (Fig 1). Technical considerations of the immunofluorescence assay limited to four the number of guinea pigs that could be tested in a single day. The dose schedule was staggered accordingly.

Peripheral blood lymphocytes--At the end of 3 weeks of aflatoxin treatment, animals were anaesthetized with carbon dioxide and bled by cardiac puncture. Ten ml of blood was collected into Vacutainer (Becton-Dickinson, Rutherford, NJ) tubes containing 143 UPS units of sodium heparin. Two ml of each blood sample were retained as whole blood for total leukocyte count, differential white blood cell count, determination of packed cell volume, and for use in bacterial binding assay. The remainder of the blood sample was processed by Ficoll-Hypaque (specific gravity = 1.080) density gradient centrifugation[13] for separation of lymphocytes and their subsequent identification by immunofluorescence.

Blood profile--Total white blood cell counts were determined on diluted blood samples (Unopettes, Becton-Dickinson, Rutherford, NY) by conventional hematologic technique. Differential white blood cell counts of 200 cells were made on air dried blood smears stained with Wright-Giemsa stain (Volu-Sol Stat Stain, Volu-Sol Medical Industries, Las Vegas, NV). Twenty microliter siliconized capillary tubes were filled with heparinized blood, centrifuged at 3000 g for 5 minutes, and the relative % packed erythrocyte volume was determined.

Identification of B and T lymphocytes by immunofluorescence--Ficoll-Hypaque purified mononuclear cells were processed for immunofluorescence at 4 C as previously described[14] using sterile saline containing 1% BSA to wash cells and to adjust cell concentrations to 1×10^7 cells/ml. B lymphocytes were identified directly by the presence of surface immunoglobulin using fluorescein isothiocyanate (FITC)-conjugated anti-guinea pig IgG (Cappel Laboratories, Cochranville, PA). T lymphocytes were identified by indirect immunofluorescence using an anti-T cell monoclonal antibody produced in mice against peripheral blood T lymphocytes (Dr. Ethan Shevach, NIH). For identification of T lymphocytes, suspensions of mononuclear cells were incubated in the presence of anti-T cell monoclonal antibody for 30 minutes at 4 C. They were then washed to remove unbound antibody and incubated in the presence of FITC-conjugated anti-mouse IgG (FaB_2 specific) (Cappel Laboratories, Cochranville, PA). Optimal dilutions of reagents were determined in preliminary studies. Appropriate negative controls were employed to standardize fluorescence on different test days. Samples were

prepared in duplicate and the relative percent of fluorescent cells was determined for 200 lymphocytes.

Bacterial binding assay--The bacterial binding assay was performed as previously described[5] with a single modification. Briefly, whole blood was washed three times with Eagle's minimum essential medium (Gibco, Grand Island, NY) containing 6% bovine serum albumin (Fraction V) (MEM-BSA). Sodium azide was omitted from the medium because it inhibited binding of bacteria by guinea pig lymphocytes.[2] Washed blood was centrifuged with formalin-fixed bacteria and washed as above to remove excess bacteria. The percentage of lymphocytes binding S schottmulleri or Y enterocolitica was determined by microscopic examination of 300 lymphocytes in stained smears.

RESULTS

Analysis of variance[15] indicated that total white blood cell counts, differential leukocyte counts, absolute total lymphocyte counts, and relative percent packed cell volume were not affected by aflatoxin at any dose level used in this experiment (results not shown).

Absolute numbers (Fig 1) of B and T lymphocytes (as detected by immunofluorescent antibody techniques), S schottmuelleri-binding and Y enterocoltica-binding lymphocytes were determined for individual guinea pigs; analysis of variance was performed following log transformation of the data. There were no significant differences ($P < 0.05$) among groups in absolute numbers of fluorescently labelled B or T lymphocytes, S schottmulleri-binding or Y enterocolitica-binding lymphocytes between nontreated animals and aflatoxin-treated animals at any dose level used in this experiment.

The absolute number of T lymphocytes identified by indirect immunofluorescence and the absolute number of S schottmulleri-binding lymphocytes is shown in Fig 1. The lymphocyte populations identified in control animals (group 1) by immunofluorescence or by adherence of S schottmulleri represented 44.7% and 40.5% of the total white blood cell count, respectively. There were no significant differences between the absolute number of S schottmulleri-binding lymphocytes and T lymphocytes from aflatoxin-treated animals in groups 1, 2, 3, or 4. However, in group 5, the absolute number of S schottmulleri-binding lymphocytes (4.39×10^9 cells/liter, 34.6% total WBC) was significantly lower ($P < 0.05$) than the absolute number of T lymphocytes (5.73×10^9 cells/liter, 45.8% total WBC).

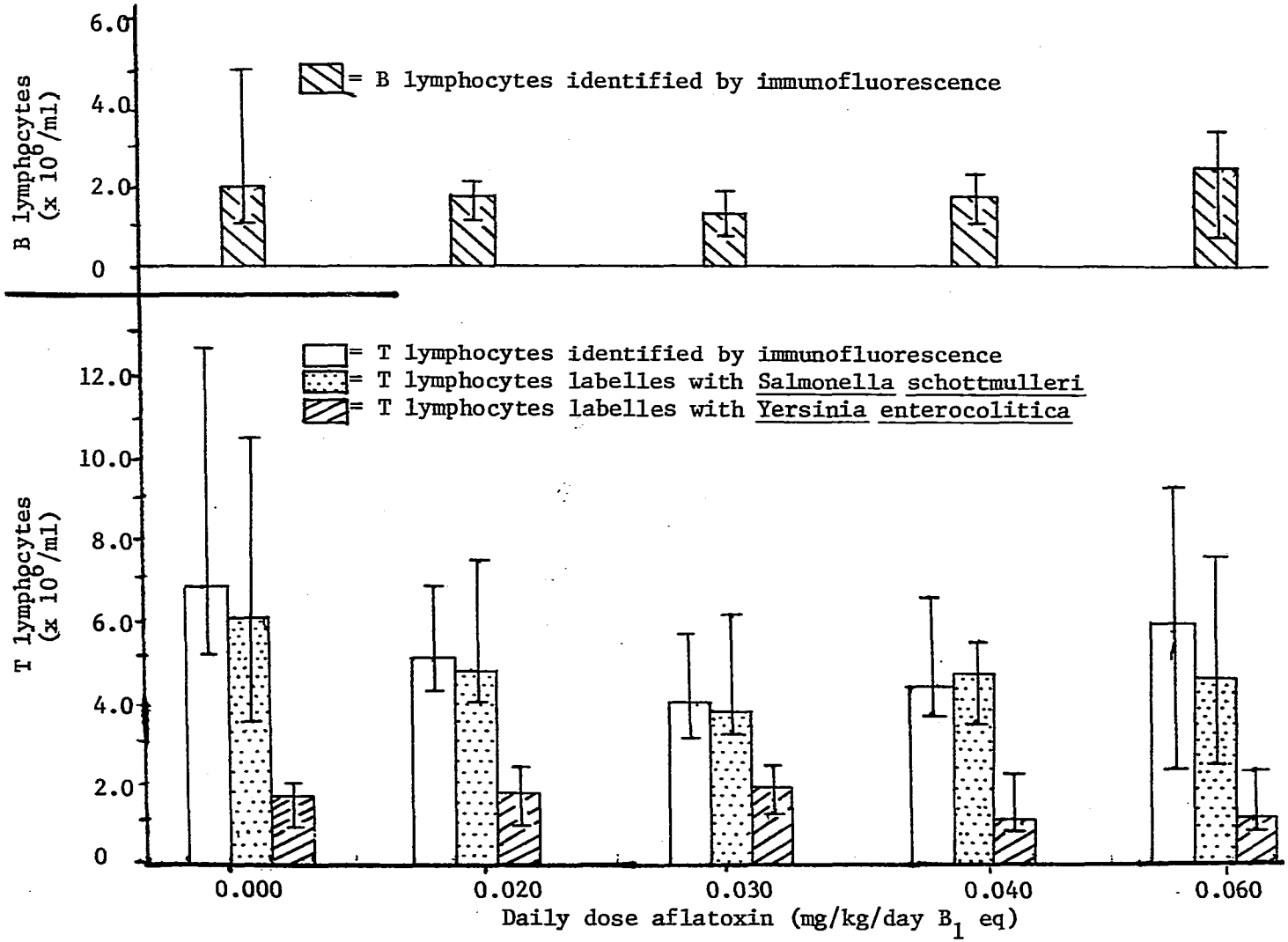

Figure 1 Mean and range of absolute numbers of T and B lymphocytes in peripheral blood of aflatoxin-treated guinea pigs

DISCUSSION

Cell-mediated immune processes, as detected by skin test responsiveness, were impaired in guinea pigs which were given 0.040 mg/kg/day B_1 eq in a dose regimen identical to the one described herein.[1] Reductions in lymphokine activity and lymphoblastogenesis were observed in guinea pigs administered 0.070 mg/kg/day B_1 eq for three weeks.[5] In the present study, aflatoxin-induced immunosuppression in guinea pigs was not the result of quantitative changes in T or B lymphocyte populations.

The present work substantiates an earlier report[6] which established that S schottmulleri was bound by the majority of guinea pig peripheral blood T lymphocytes. In group 1, the absolute number of S schottmulleri-binding lymphocytes very closely approximated the number of T lymphocytes identified by immunofluorescence. There were no differences between the number of S schottmulleri-binding lymphocytes and T lymphocytes identified by immunofluorescence for aflatoxin treatment groups 2, 3, and 4. However, at the highest dose level used (0.060 mg/kg/day B_1 eq, group 5), the absolute number of S schottmulleri-binding lymphocytes was significantly lower ($P < 0.05$) than the absolute number of T lymphocytes identified by immunofluorescence. These data suggest that at this level of aflatoxin ingestion, a portion of T lymphocytes that were identified by immunofluorescence were altered by aflatoxin in some way so as to preclude binding of S schottmulleri. Since immature T lymphocytes obtained from thymic tissue did not bind S schottmulleri,[6] perhaps aflatoxin caused an increased turnover of T lymphocytes which were unable to bind S schottmulleri. The number of

Y enterocolitica-binding lymphocytes was similar to values obtained in earlier studies.[6] The absolute number of Y enterocolitica-binding lymphocytes was not affected by aflatoxin at any dose level used in this experiment. T lymphocytes binding S schottmulleri or Y enterocolitica thus appeared to represent mutually exclusive lymphocyte subsets, with differing susceptibilities to aflatoxin.

Aflatoxin did not affect the number of T and B lymphocytes in peripheral blood. Its immunosuppressive effects[10] must relate to functional or maturational aspects of lymphocyte populations, impaired interactions between cell types involved in cell-mediated immune processes, or some other as yet unidentified mechanism for immunologic regulation of cell mediated immune responses.

SUMMARY

Aflatoxin did not affect the total white blood cell count, differential leukocyte count, absolute lymphocyte count, or relative percent packed erythrocyte volume of guinea pigs which received as much as 0.060 mg/kg/day B_1 eq for three weeks. No change in absolute numbers of peripheral blood T or B lymphocytes was observed in any group of aflatoxin-treated guinea pigs when immunofluorescence was used to identify lymphocyte popluations. Aflatoxin did decrease the number of lymphocytes identified by adherence of Salmonella schottmulleri in the highest dose group (0.060 mg/kg/day B_1 eq) relative to the number of T lymphocytes identified by immunofluorescence. In this group, the number of S schottmulleri-binding lymphocytes (tentatively identified as T lymphocytes) was significantly lower than the number of T lymphocytes identified by immunofluorescence.

Aflatoxin treatment did not cause changes in absolute numbers of lymphocytes or shifts in lymphocyte populations as determined by immunofluorescence. After high levels of aflatoxin were ingested by guinea pigs, lymphocytes identified as T lymphocytes by immunofluorescence became refractory to identification by S schottmulleri.

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SUMMARY AND DISCUSSION

This research was part of a current project at the National Animal Disease Center designed to investigate mechanisms of immunosuppression associated with ingestion of aflatoxin. Attempts were made to: (1) define the dose at which aflatoxin consistently induces suppression of cellular immune responses in guinea pigs and (2) characterize the leukocyte populations functionally and numerically.

In general, aflatoxin affected skin test responsiveness and weight gain in a dose-related manner. Guinea pigs which received at least 0.040 mg/kg/day B_1 eq for three weeks had reduced delayed type hypersensitivity skin reactions when tested with homologous Nocardia asteroides sensitin. Reduced weight gains occurred in guinea pigs that received 0.020 mg/kg/day B_1 eq. Elevation of serum bile acids indicated hepatotoxic effects of aflatoxin at very low levels of ingestion. Glycocholic acid was the most sensitive indicator of hepatotoxic effects of aflatoxin, with elevated serum levels occurring in guinea pigs given the lowest dose (0.010 mg/kg/day B_1 eq). Aflatoxin-induced alterations of serum levels of bile acids were seen in guinea pigs which had normal weight gains and skin test responses.

A passive transfer model was employed to study T lymphocytes and macrophages in the skin test response. Experimental groups were designed to test the role of T lymphocytes and macrophages in cellular immune responses of aflatoxin-treated guinea pigs. Skin test responses indicated that aflatoxin impaired the function of both these cell types, but differ-

ences were not statistically significant. This may be attributed to individual variation in susceptibility to aflatoxin of guinea pigs within groups and limitations inherent in the passive transfer assay. Further, 40% of the aflatoxin-treated recipients died following intraperitoneal injection of PEC from guinea pigs sensitized to Nocardia asterodies, with the result that only the most aflatoxin-resistant guinea pigs lived to be skin tested.

Interest in differentiating guinea pig lymphocyte populations in peripheral blood resulted in adapting for use in guinea pigs a bacterial binding assay currently used to identify human and murine lymphocytes. Fifty-three bacterial strains and ten yeasts were screened for this purpose. Those which consistently demonstrated binding by a portion of guinea pig peripheral blood lymphocytes (PBL) were evaluated further. Lymphocytes from spleen, thymus, and lymph node as well as T-enriched preparations of peripheral blood lymphocytes were tested in an effort to identify the lymphocyte subset populations labelled with bacteria.

Lymphocytes obtained from lymphoid tissues had different patterns of binding than PBL. Significantly, S schottmulleri which was bound by approximately half of PBL, was not bound by guinea pig thymocytes and was bound by fewer lymphocytes from spleen and lymph node relative to PBL. Yersinia enterocolitica and B melitensis were bound by more lymphocytes from lymphoid tissues relative to binding by PBL. A hemolytic strain of E coli which was not bound by guinea pig PBL was bound by lymphocytes from lymphoid tissue.

Disparate binding patterns were observed with preparations of "T-enriched" lymphocytes obtained by passage of PBL over nylon wool adherence columns. Salmonella schottmulleri and Y enterocolitica demonstrated increased binding by T-enriched PBL, suggesting that these bacteria label a subpopulation of guinea pig T lymphocytes in peripheral blood. Decreased binding by T-enriched PBL relative to untreated PBL indicated that B melitensis was bound by guinea pig B lymphocytes. The identity of guinea pig lymphocyte populations labelled by S schottmulleri and B melitensis using T-enriched PBL is in accord with results from experiments using these bacteria to identify human lymphocytes.[45]

Peripheral blood T and B lymphocytes from nontreated and aflatoxin-treated guinea pigs were enumerated using two methods. Specific antibody directed against surface immunoglobulin and a monoclonal antibody with specificity for peripheral blood T lymphocytes were used to identify B and T lymphocytes, respectively, by immunofluorescence. Salmonella schottmulleri and Y enterocolitica were used to label T lymphocytes in aflatoxin-treated guinea pigs to detect possible changes in T lymphocyte subpopulations.

The highest dose, 0.060 mg/kg/day B_1 eq, was sufficient to cause immunosuppression in the study described in Section I; yet the absolute numbers of B and T lymphocytes identified by immunofluorescence were unaffected by aflatoxin at this dose level. Similarly, the numbers of S schottmulleri-positive and Y enterocolitica-positive lymphocytes from aflatoxin-treated guinea pigs were not significantly different from those

of nontreated guinea pigs. The blood profile, including total white blood cell count, absolute lymphocyte count, differential leukocyte count, and relative percent packed erythrocyte volume, of aflatoxin-treated guinea pigs remained stable over the range of doses used in this experiment; it was of no value in detecting aflatoxin-induced alterations of cell types involved in cellular immune processes.

Certain of the results summarized above can be discussed by considering maturational and/or functional aspects of lymphocytes in normal and aflatoxin-treated guinea pigs. Recently, the concept of "active" T cells has been applied to guinea pig PBL. Approximately 35% of all guinea pig PBL will form heterologous red blood cell rosettes immediately; these lymphocytes comprise a T lymphocyte subpopulation which participates in cell-mediated immune processes. An additional 40-50% PBL will form rosettes after prolonged cold incubation. The population of guinea pig lymphocytes which binds S schottmulleri bears some similarities to PBL designated as "active" T lymphocytes. Of interest to the present discussion is the distribution of "active" T lymphocytes in lymphoid organs. They represent 32% of lymphoid cells in peripheral blood, 29% in lymph nodes, 13% in spleen, 10% in thymus, and 0% in bone marrow.[46] Compare these results with results of binding of S schottmulleri by lymphocytes from peripheral blood (49%), lymph node (22%), spleen (18%), and thymus (0%). Binding of S schottmulleri was inhibited in the presence of sodium azide as is the formation of heterologous red blood cell T rosettes.[41] Finally, PBL bind S schottmulleri during a six-minute low speed centri-

fugation without prior incubation. Partial or complete identity of S sschottmulleri-binding lymphocytes and "active" T cells is probable.

The numbersof S schottmulleri-positive lymphocytes very closely approximated the numbers of T lymphocytes identified by immunofluorescence in nontreated guinea pigs. This correlation was maintained for all except the highest dose level of aflatoxin. Guinea pigs which received 0.060 mg/kg/day B_1 eq demonstrated reduced numbers of lymphocytes identified by S schottmulleri relative to T lymphocytes identified by immunofluorescence. Since the absolute number of lymphocytes was unchanged at this dose level, aflatoxin must have altered the ability of T lymphocytes to bind S schottmulleri. Aflatoxin-induced differences in binding affinity may reflect maturational differences among lymphocytes from nontreated and aflatoxin treated guinea pigs. It was observed earlier that thymocytes do not bind S schottmulleri. Perhaps aflatoxin causes increased turnover of T lymphocytes in peripheral blood and, consequently, a shift toward immature T lymphocytes incapable of binding S schottmulleri. If S schottmulleri-binding lymphocytes are partially or wholly analagous to "active" T lymphocytes, the decrease in S schottmulleri-binding lymphocytes observed in aflatoxin-treated guinea pigs may have functional significance with regard to aflatoxin-induced immunosuppression.

Aflatoxin-induced suppression of lymphokine production and/or activity has been proposed to account for the mycotoxin's suppressive effect on skin test responsiveness, lymphoblastogenesis, and lymphokine activity.[30] Such a mode of action is feasible in view of aflatoxin's

inhibition of protein synthesis. Both lymphoblastogenesis and elaboration of lymphokine, in particular migration inhibition factor, require active protein synthesis.[33]

This research supports a functional hypothesis to explain aflatoxin's suppression of cell-mediated immune responsiveness while suggesting that factors unrelated to T lymphocytes are also involved. Results of the passive transfer study indicated that aflatoxin exerted a suppressive action on T lymphocytes; in this model the effect of aflatoxin on macrophages could be distinguished from nonspecific alteration by aflatoxin of host resistance. However, in the passive transfer experiment described herein, aflatoxin-treated recipients demonstrated reduced skin test responses despite the injection of T lymphocytes from untreated donor guinea pigs sensitized to N asteroides. Perhaps, immunocompetent T lymphocytes present in PEC from treated guinea pigs were introduced into a suppressive environment in the aflatoxin-treated recipient guinea pig. Very probably, much of aflatoxin's immunosuppressive activity can be related to a more basic action of aflatoxin on cellular DNA[34] with resultant inhibition of protein synthesis.

The findings in this study have provided information about aflatoxin's immunosuppressive action in guinea pigs. The identification of bacteria which bind to guinea pig lymphocytes facilitated enumeration of lymphocyte populations in aflatoxin-treated guinea pigs. Further characterization of guinea pig lymphocyte populations and subpopulation will be possible using these bacterial markers.

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... and so there ain't nothing more to write about, and I am rotten glad of it, because if I'd a knowed what a trouble it was to make a book, I wouldn't a tackled it and I ain't agoing to no more.

--Mark Twain

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