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# Hematological Toxicology following Embryonic Exposure to Aflatoxin-$B_1$ (41674)

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*Abstract.* The influence of embryonic exposure to aflatoxin-$B_1$ (AF-$B_1$) upon the erythroid system of the maturing chicken was examined using a variety of assays. Since the chick embryo is known to possess mixed-function oxidase activity, this animal serves as an excellent model system for studies of human fetal toxicology. AF-$B_1$ (0.1 $\mu$g) was administered to either 6- or 12-day embryos by the air sac method. This level of AF-$B_1$ was highly mutagenic and was found to induce an average of 10.6 sister chromatid exchanges (SCEs) per cell compared with 1.8 SCEs per cell for the acetone control solvent. Despite selection against treated embryos through acute and chronic embryonic toxicity, hatched chicks from AF-$B_1$ treatment groups exhibited erythroid anemia when compared to the acetone controls. Cell count, hematocrit, and hemoglobin concentration were all significantly reduced in the 12-day AF-$B_1$ treatment groups compared with controls. Both sexes were equally affected. While the number of peripheral erythrocytes was reduced following exposure to AF-$B_1$, the differentiation status of erythrocytes was apparently unaltered. Mean cell volume, percentage of circulating reticulocytes, and incidence of an erythroid differentiation marker, chicken fetal antigen, were parameters in which no treatment effects were observed. An apparent maturation effect was noted since adult hematocrits were similar between control and treatment groups. Possible explanations for this age effect are discussed. The ability to detect significant posthatch erythroid toxicity following embryonic exposure to mutagenic levels of AF-$B_1$ suggests the importance of this general approach to perinatal carcinogenic evaluation.

The need for a comprehensive approach for determining the genotoxicity of environmental chemicals has led to the development of a variety of assays. While many are *in vitro* assays such as the Ames test (1) and the CHO test for sister chromatid exchange (SCE) (2, 3), others involve *in vivo* assays usually performed in rodents (4). Most assay systems employ a single genetic end-point such as chromosome aberrations, SCE, and point mutations. While these assays can be used to determine the relative abilities of compounds to induce a particular genetic alteration, the actual biological consequences of exposure to such compounds often remains undetermined. It is only by using a spectrum of toxicological assays that a full assessment of the potential health risk following exposure to an environmental agent may be achieved.

Even with the present variety of available assays, the ability to predict the long-term biological hazards (cancer, birth defects, immune suppression) resulting from prenatal exposure to compounds is limited. Indeed, prior experiences with compounds such as diethylstilbestrol, thalidomide, arsenic, diethylnitrosamine, and aflatoxin-$B_1$ suggest that this is an important area of toxicological screening (5, 6).

The purpose of this communication is to describe one aspect of a comprehensive approach for assessing long-term health risk from embryonic exposure to environmental compounds. While the overall experimental protocol combines genetic, hematopoietic, and reproductive toxicology in one experimental system, this communication will emphasize results obtained from analysis of the erythroid system. The animal model employed, the chick embryo, has a number of features attractive for studies in mutagenesis, hematolymphopoiesis, toxicology, and oncogenesis (7–13), as well as possessing properties similar to those found in the human embryo. For example, in both the chick and human there is the early appearance of a mixed-function oxidase (MFO) system capable of activating and detoxifying xenobiotics and the development of inducibility in the fetal hepatic MFO system (9, 14, 15). The early tissue differentiation patterns for these two species are very similar (16). Such characteristics are lacking in available *in vivo* animal models. Since embryonic MFO activity is present in



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the chicken, aflatoxin-$B_1$, an indirect-acting environmental mutagen–carcinogen, was selected for initial analysis in this test system.

**Materials and Methods.** The embryos used in this study were obtained from fertile Cornell K-strain eggs. Characteristics of this strain include high resistance to Marek's disease, uniform egg size, and high rate of lay (17). Blood was collected by venipuncture in either crystalline EDTA for hematology or 10% sodium EDTA for microcytotoxicity assays. Cell count, hematocrit, mean cell volume, and hemoglobin concentration of 12-day-old chicks were determined with the aid of a ZBI counter, a hemoglobinometer, and a McV/Hct Data Pack (Coulter Electronics). Four-C (Coulter) was used to calibrate all instruments prior to assay. The percentage of peripheral reticulocytes was determined by methylene blue staining as previously described (18). Five hundred cells from individual samples were scored to determine the incidence of circulating reticulocytes.

The incidence of peripheral red blood cells (PRBCs) bearing chicken fetal antigen (CFA) in 11-week-old chickens was assayed by complement-mediated microcytotoxicity as previously described (19). Blood samples were rinsed three times in 0.1 *M* phosphate-buffered saline (PBS), pH 7.2. The buffy coat was removed by aspiration and a 0.033% dilution of packed RBCs in PBS was used in the assay. One microliter of rabbit anti-CFA diluted in PBS to 1/16 was reacted with 1 μl of the PRBC solution from 11-week-old chicks for 30 min at room temperature. Following incubation with guinea pig complement (5 μl) (diluted 1/20 in PBS) for 1 hr at room temperature, the degree of cell lysis was determined by comparing free nuclei to intact cells (19). CFA-specific microcytotoxicity was calculated using the following formula:

$$\%\ \text{specific cytotoxicity} = \frac{\%\ \text{cytotoxicity in test well} - \%\ \text{control cytotoxicity}}{100 - \%\ \text{control cytotoxicity}} \times 100$$

Adult chicken hematocrits were obtained using Wintrobe tubes (Clay Adams). Hematocrits were read following a 20-min centrifugation at 800*g*.

Aflatoxin $B_1$ (AF-$B_1$) and spectrophotometric grade acetone were obtained from Aldrich and Mallinckrodt, respectively. A 0.1-μg dose of AF-$B_1$ in 10 μl of acetone or 10 μl of acetone alone was administered to 6- or 12-day-old chick embryos. The air cell method of application in which the solution is dropped onto the inner shell membrane through a hole above the air shell was used in all assays (7). The extent of genotoxicity was determined by detecting the levels of sister chromatid exchanges (SCEs) in cells of AF-$B_1$-exposed embryos. The dosage of AF-$B_1$ (0.1 μg) was selected from previous toxicity and mutagenicity trials (9). Briefly, embryos were injected with 100 μl (1 mg) of 5-bromodeoxyuridine (BrdU) to label chromosomal DNA over two cell cycles (24 hr). AF-$B_1$ or the solvent control was applied to eggs 1 hr following the BrdU injection. Two hours prior to harvest, embryos were exposed to 100 μl Colcemid (50 μg) for mitotic arrest. The embryos were excised, treated in 0.9% sodium citrate for 30 min, and fixed in 1:3 acetic ethanol. Chromosome preparations were made by a modified solid tissue technique (8). SCEs were scored in the macrochromosomes from 50 cells per embryo. All assays were performed on individual samples. Data were statistically analyzed using a two-tailed Student's *t* test on actual and transformed ($\log_{10}$) data.

**Results.** The level of aflatoxin-$B_1$ used in these experiments is known to be genotoxic. The baseline SCE level was found to be 1.8 per cell in the presence of the acetone solvent alone. With 0.1 μg of AF-$B_1$ the frequency of SCE was 10.6 per cell. Exposure to AF-$B_1$ and/or acetone produced acute and chronic embryonic mortality. As a result, exposure of chick embryos to these treatments resulted in a selection of tolerant embryos. Despite this inherent selection for embryonic survivors, the hatched chickens demonstrated several changes in erythroid characteristics.

Blood parameters were obtained from 12-day-old chickens and included cell count (Table I), hematocrit (Table I), mean cell volume (Table II), hemoglobin concentration (Table III), and percentage circulating reticulocytes (Table IV). Embryos exposed to AF-$B_1$ at 12 days of development had a significantly reduced cell count, hematocrit, and hemoglobin

TABLE I. CELL COUNT AND HEMATOCRIT IN 12-DAY-OLD CHICKS

| | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Cell count | | Hematocrit | | | Cell count | | Hematocrit | |
| Treatment | $N$ | Mean[a] | (SD) | Mean | (SD) | $N$ | Mean | (SD) | Mean | (SD) |
| 6-AF-$B_1$ | 22 | 221.59 | (38.2) | 29.70 | (5.1) | 26 | 225.15 | (40.9) | 29.88 | (5.9) |
| 6-Acetone | 20 | 233.85 | (27.0) | 31.96 | (4.8) | 31 | 253.26 | (48.4) | 34.40 | (7.2) |
| 12-AF-$B_1$ | 28 | 226.61 | (35.7)* | 30.28 | (4.7)** | 24 | 226.29 | (30.1)* | 29.88 | (3.9)** |
| 12-Acetone | 27 | 289.48 | (57.6)* | 38.11 | (8.4)** | 29 | 268.24 | (54.9)* | 35.88 | (8.0)** |

[a] $\times 10^{-2}$ in 0.5 ml of a 1/50,000 whole blood dilution.
* A 12-day pairwise comparison of cell count within each sex is significantly different at $P \leq 0.01$ using a two-tailed Student's $t$ test.
** A 12-day pairwise comparison of hematocrit within each sex is significantly different at $P \leq 0.01$ using a two-tailed Student's $t$ test.

concentration when compared to the acetone controls. Both sexes were equally affected. However, mean cell volume and the percentage of circulating reticulocytes in the blood were not altered by the treatment. While the same patterns were observed in the 6-day exposure groups, the differences were not significant.

In contrast, measurements of two parameters related to erythroid differentiation and development obtained from older chickens revealed no differences between the acetone control and AF-$B_1$ treatment groups. The incidence of an oncodevelopmental antigen, chicken fetal antigen (CFA), on peripheral RBCs was determined by complement-mediated microcytotoxicity. This antigen system is known to undergo developmental loss from PRBCs during posthatch maturation of the chicken. In particular, the incidence of CFA-positive PRBCs has been shown to decline between 40 and 120 days of age (20). In the 11-week-old chicken, no differences were observed between control and treatment groups with regard to the incidence of CFA in peripheral blood (Table V). Furthermore, hematocrit levels that were significantly different in the young chick exhibited no differences in the 26-week-old adult (Table VI).

**Discussion.** The potential of the developing chicken as a model for comprehensive testing in toxicology is indicated by several observations. The presence of baseline mixed-function oxidase (MFO) activity ensures the metabolism of promutagens to active mutagenic metabolites as well as detoxification of certain compounds (9, 15, 21, 22). This resembles the situation for the human fetus, which exhibits precocious MFO activity (23). In the present study, it was possible to hatch chicks from embryos even after exposure to doses of AF-$B_1$ that induced high frequencies of SCE. Since

TABLE II. MEAN CELL VOLUME (MCV) IN 12-DAY-OLD CHICKS

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Treatment | $N$ | Mean[a,b] | (SD) | $N$ | Mean | (SD) |
| 6-AF-$B_1$ | 22 | 134.68 | (8.5) | 26 | 132.50 | (7.0) |
| 6-Acetone | 20 | 137.35 | (14.3) | 31 | 135.68 | (6.4) |
| 12-AF-$B_1$ | 28 | 133.64 | (6.8) | 24 | 132.58 | (8.6) |
| 12-Acetone | 27 | 131.26 | (6.6) | 29 | 134.52 | (7.0) |

[a] No comparisons were statistically different at $P \leq 0.01$ using a two-tailed Student's $t$ test.
[b] Expressed as $\mu^3$.

TABLE III. HEMOGLOBIN CONCENTRATION IN 12-DAY-OLD CHICKS

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Treatment | $N$ | Mean[a] | (SD) | $N$ | Mean | (SD) |
| 6-AF-$B_1$ | 22 | 12.89 | (2.3) | 26 | 13.29 | (2.6) |
| 6-Acetone | 20 | 14.15 | (2.0) | 32 | 13.69 | (2.8) |
| 12-AF-$B_1$ | 28 | 12.75 | (1.4)* | 24 | 12.80 | (1.4)* |
| 12-Acetone | 27 | 14.81 | (2.8)* | 29 | 14.59 | (2.4)* |

[a] Expressed as g/100 ml blood.
* A 12-day pairwise comparison within each sex is significantly different at $P \leq 0.01$ using a two-tailed Student's $t$ test.

TABLE IV. PERCENTAGE OF CIRCULATING RETICULOCYTES IN THE 12-DAY-OLD CHICKEN

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Treatment | $N$ | Mean[a] | (SD) | $N$ | Mean | (SD) |
| 6-AF-$B_1$ | 36 | 9.73 | (3.5) | 43 | 6.56 | (3.0) |
| 6-Acetone | 33 | 9.14 | (3.3) | 39 | 8.01 | (3.1) |
| 12-AF-$B_1$ | 32 | 7.02 | (3.1) | 27 | 7.79 | (2.8) |
| 12-Acetone | 30 | 5.94 | (5.9) | 30 | 6.38 | (3.9) |

[a] No comparisons were statistically different at $P \leq 0.01$ using a two-tailed Student's $t$ test.

TABLE VI. HEMATOCRIT IN ADULT CHICKENS

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Treatment | $N$ | Mean[a] | (SD) | $N$ | Mean | (SD) |
| 6-AF-$B_1$ | 11 | 41.46 | (2.7) | 11 | 36.00 | (2.9) |
| 6-Acetone | 12 | 41.75 | (4.2) | 11 | 37.09 | (3.2) |
| 12-AF-$B_1$ | 12 | 40.08 | (3.3) | 9 | 36.44 | (3.3) |
| 12-Acetone | 11 | 43.27 | (2.5) | 12 | 37.21 | (2.1) |

[a] No comparisons were statistically different at $P \leq 0.01$ using a two-tailed Student's $t$ test.

SCE is an indicator of DNA damage, the actual extent of mutation is not known. The viability of these chicks permitted evaluation of long-term health risk following a single embryonic exposure to a known mutagen–carcinogen, AF-$B_1$, and to a somewhat toxic nonmutagenic solvent, acetone. The present communication describes the long-term alteration of erythroid hematology.

Embryonic exposure to AF-$B_1$ resulted in alteration of several parameters relating to erythroid development and differentiation. While no differences were detected in the nature of circulating erythrocytes, embryonic exposure to AF-$B_1$ resulted in a subsequent reduction in the numbers of circulating RBCs, hematocrits, and hemoglobin levels in the blood of hatched chicks. These findings are similar to those from studies in which chickens were treated after hatching with prolonged exposure to AF-$B_1$ (24). However, in the present study erythroid alteration was detected long after a single embryonic exposure to AF-$B_1$ without the necessity of posthatch exposure. Since AF-$B_1$ active metabolities are short-lived, the alterations we observed in the postnatal erythroid system probably resulted from toxicity (and perhaps mutagenicity) to the early cell populations from the metabolic activation of AF-$B_1$. It has recently been shown that an initial embryonic RBC population is lost and replaced by a second embryonically derived population starting at 10–12 days of development (25). Our mutagenic treatment occurred at the time of production of the second embryonic cell population that populates the bone marrow for future erythroid cell production.

TABLE V. INCIDENCE OF CHICKEN FETAL ANTIGEN ON 11-WEEK CHICKEN RBCs AS DETERMINED BY MICROCYTOTOXICITY

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Treatment | $N$ | Mean[a,b] | (SD) | $N$ | Mean | (SD) |
| 6-AF-$B_1$ | 20 | 83.0 | (17.3) | 16 | 81.8 | (19.5) |
| 6-Acetone | 20 | 87.4 | (10.1) | 18 | 81.4 | (14.9) |
| 12-AF-$B_1$ | 21 | 82.1 | (12.8) | 21 | 81.6 | (13.8) |
| 12-Acetone | 18 | 90.5 | (8.5) | 21 | 87.6 | (8.4) |

[a] Expressed as percentage of peripheral RBCs bearing chicken fetal antigen.

[b] No comparisons were statistically different at $P \leq 0.01$ using a two-tailed Student's $t$ test.

The lack of difference observed in the incidence of CFA or in adult hematocrits was in contrast to RBC parameters in the young chick. Two explanations appear likely: (i) The erythroid anemia induced by AF-$B_1$ may be gradually reversed during maturation of the chicken; or (ii) selection against anemic birds during the growing period could result in a higher mortality that could influence the adult data. The known negative impact of AF-$B_1$ on the immune system could also contribute to the latter hypothesis (26, 27). Further investigation should help distinguish between these possibilities.

In conclusion, we have demonstrated that a single embryonic exposure to a known indirect-acting mutagen–carcinogen, AF-$B_1$, resulted in dramatic posthatch alterations of the erythroid system. These results suggest that interruption of normal processes during critical periods of development can lead to substantive cellular alterations in the growing animal. When early exposure to a compound,

regardless of mutagenic potential, results in altered differentiation or development, potential impact of this alteration on the overall health of the animal is likely, and therefore, a critically important consideration. Although posthatch alteration of the erythroid system alone may or may not pose an imminent health risk to the chicken, future reports will describe similar long-term effects on the immune system and resistance to disease. Therefore, this general approach to perinatal toxicology screening appears promising.

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