Aflatoxin B₁-DNA Adduct Formation and Mutagenicity in Livers of Neonatal Male and Female B6C3F1 Mice

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Exposure to genotoxic chemicals at a young age increases cancer incidence later in life. Aflatoxin B₁ (AFB₁) is a potent genotoxin that induces hepatocellular carcinoma (HCC) in many animal species and in humans. Whereas adult mice are insensitive to aflatoxin-induced carcinogenesis, mice treated with AFB₁ shortly after birth develop a high incidence of HCC in adulthood. Furthermore, the incidence of HCC in adult male mice treated as infants is much greater than in females, reasons for which are unclear. In this study, treatment with AFB₁ produced similar levels of DNA damage and mutations in the liver of newborn male and female gpt delta B6C3F1 mice. Twenty-four hours after dosing with AFB₁ (6 mg/kg), the highly mutagenic AFB₁-FAPY adduct was present at twice the level of AFB₁-N⁷-guanine in liver DNA of males and females. A multiple dose regimen (3 × 2 mg/kg), while delivering the same total dose, resulted in lower AFB₁ adduct levels. Mutation frequencies in the gpt transgene in liver were increased by 20- to 30-fold. The most prominent mutations in AFB₁-treated mice were G:C to T:A transversions and G:C to A:T transitions. At this 21-day time point, no significant differences were found in mutation frequency or types of mutations between males and females. These results show that infant male and female B6C3F1 mice experience similar amounts of DNA damage and mutation from AFB₁ that may initiate the neoplastic process. The gender difference in the subsequent development of HCC highlights the importance of elucidating additional factors that modulate HCC development.

Key Words: aflatoxin; neonatal mouse; hepatocarcinoma; mutation; gpt delta mouse.

Despite an abundance of experimental data, the mechanistic basis for the high sensitivity of infant animals to AFB₁ and other genotoxic carcinogens is not fully understood. A related unanswered question is why exposure during infancy induces higher incidence of HCC in males than in females in adulthood. Treatment of newborn B6C3F1 mice with single or multiple doses of AFB₁ induced HCC by 82 weeks of age in >90% in males compared with <10% of similarly treated females (Vesselinovitch et al., 1972). Similar experiments showed that female mice treated with diethylaminolesamine (DEN) develop HCC much less frequently than males (Nakatani et al., 2001). Two possible explanations are: (1) in female mice, carcinogen activation may be less effective or inactivation and DNA repair more effective, resulting in less genetic damage and thus fewer tumor-initiating mutations than male mice, or (2) similar amounts of genetic damage may occur in both sexes and subsequent hormonal and/or other environmental factors differentially modulate HCC development. Prior studies showed that hormonal status had a profound effect on tumor development in animals treated during the neonatal period. For example, orchidectomy of neonatal male mice dosed with DEN delayed tumor onset and reduced tumor yield (Vesselinovitch, 1990). Estrogen-mediated inhibition of inflammatory responses in the liver following DEN treatment has also been shown to attenuate the postinitiation development of HCC in female mice (Naugler et al., 2007). Little information exists, however, on the relative importance of genotoxic damage in neonates as compared with postinitiation events that determine differences in HCC incidence between males and females later in life.

In mice, AFB₁ is metabolized by cytochromes P450 1A2 and 3A4 to the 8,9-epoxide, which reacts with cellular DNA, producing the predominant AFB₁-N⁷-guanine adduct (Eaton and Gallagher, 1994). DNA adduct formation by the AFB₁-8,9-epoxide can be diminished by formation of AFB₁-glutathione conjugates, mediated by alpha class glutathione-S-transferases (GSTs) (Hayes et al., 1992). Increased expression of GSTs during the postnatal period is...
believed to be largely responsible for the diminished sensitivity of older mice (Shupe and Sell, 2004). Furthermore, in the rat, it has been shown that chemoprotective chemicals such as dithiole-thiones, that induced GSTs and inhibited formation of AFB1-DNA adducts also reduced the number of hepatic preneoplastic lesions and prevented tumor development (Roebuck et al., 2003).

Importantly, epidemiological studies have validated aflatoxin-DNA adducts as biomarkers of risk of HCC from aflatoxin exposure (Groopman et al., 2002, 2008). An additional molecular connection between AFB1 exposure and human HCC was established by the observation that G:C to T:A transversions characteristic of those induced by AFB1-DNA adducts were found at high frequencies in codon 249 of the p53 gene in HCC of patients residing in geographical areas in which AFB1 exposure is an established risk factor (Hussain et al., 2007).

The overall objectives of our study were to quantify aflatoxin adduct levels, characterize the frequency and spectrum of mutations induced in the liver of male and female gpt delta B6C3F1 mice, and assess relationships of these parameters to the known sensitivity of this strain of mice to AFB1-induced HCC. The mutagenic potency of AFB1 is well established in bacteria and mammalian cells, and G:C to T:A transversions are the most frequent base substitution mutation induced by AFB1-DNA adducts. A prior study with similar objectives carried out in Big Blue lacI transgenic mice showed that AFB1 is a potent liver mutagen in neonatal animals, but much less potent in the adult (Chen et al., 2010), in which fewer AFB1-DNA adducts were previously found (Shupe and Sell, 2004). We carried out the study in the B6C3F1 mouse, which is extensively used as a bioassay animal for testing chemicals for carcinogenic activity and for which a large carcinogenesis literature exists. This work is an early stage of a continuing effort to define biochemical markers related to age, gender, and strain capable of identifying key biochemical processes that underlie sensitivity and resistance to carcinogens.

**MATERIALS AND METHODS**

**Caution.** Aflatoxin B1 is toxic, mutagenic and carcinogenic. This compound should be handled using appropriate precautions.

**Animals.** C57BL/6 gpt delta transgenic mice were obtained from Takehiko Nohmi (Nohmi et al., 1996). The gpt delta B6C3F1 mice used in our experiments were generated by breeding female gpt delta C57BL/6J mice, which harbor an estimated 80 copies of the gpt gene on chromosome 17 (Nohmi et al., 1996), with male C3H/HeJ mice purchased from the Jackson Laboratories (Bar Harbor, ME). All experiments were conducted in accordance with protocols approved by the MIT Committee on Animal Care.

**AFB1 treatment for adduct analysis.** In the time course study of adduct formation, male and female transgenic B6C3F1 mice were injected ip on postnatal day 4 with a single dose of 6 mg/kg AFB1 in 10 l of dimethyl sulfoxide (DMSO), both obtained from Sigma-Aldrich (St Louis, MO). Mice were euthanized and livers were collected 2, 4, 8, 12, 24, and 48 h after treatment; livers were collected from a minimum of three male and three female animals at each time point. To assess adduct formation induced by a multiple dose regimen, male and female mice were injected ip on postnatal days 4, 7, and 10 with a dose of 2 mg/kg AFB1 in 10 l DMSO. Twenty-four hours after the last dose, animals were euthanized and livers were collected from 13 mice of each sex.

**AFB1 treatment for gpt mutation assay.** Parallel experiments were conducted in which the single- or multiple-dose AFB1 regimens were employed. In the former protocol, as described above male and female mice were injected ip on postnatal day 4 with a single dose of 6 mg/kg AFB1 in 10 l DMSO or 10 l DMSO alone. Animals were euthanized 21 days after the single dose was administered and livers were collected from a minimum of four male and four female mice. In the second treatment schedule, male and female mice were injected ip on postnatal days 4, 7, and 10 with either 2 mg/kg AFB1 in 10 l DMSO or DMSO alone. These animals were euthanized 21 days after the last dose and livers were collected from a minimum of four male and four female mice.

**Isolation of liver DNA and hydrolysis of AFB1-DNA adducts.** DNA was isolated from livers of AFB1-treated mice and DNA isolated using previously described procedures (Groopman et al., 1980; Kensler et al., 1986). For adduct analysis, AFB1-DNA adducts were released by hydrolysis in 1.0 N HCl at 95°C for 15 min (Groopman et al., 1981). Internal 15N2-guanine-derived standards for both AFB1-N7-guanine and AFB1-FAPY were added after hydrolysis of AFB1-DNA adducts to permit quantitative analysis by isotope dilution mass spectrometry.

**DNA adduct analysis.** Ultra-high performance liquid chromatography (UPLC) was used to separate AFB1-DNA adducts prior to measurement by isotope dilution mass spectrometry (MS) as previously described (Egnier et al., 2006). UPLC was carried out on an Acquity C18 1.7 μm 1.0 × 150 mm column, and the composition of the initial mobile phase was 14% methanol, 1% acetonitrile, 0.1% formic acid, and 85% water. An 8-min linear gradient was employed, reaching a final mobile phase consisting of 37% methanol, 2% acetonitrile, 0.1% formic acid, and 61% water. Flow rate was 120 μl/min. The hydrolyzed DNA solution was diluted into the initial UPLC mobile phase and then injected for MS/MS analysis of AFB1-DNA adduct levels. The protonated parent ion of the AFB1-N7-guanine adduct (m/z 480.1) was selected and subjected to collision-induced fragmentation producing a m/z 152 product ion that was monitored to quantify adduct levels. The AFB1-FAPY adduct was quantified by selection of the m/z 498 parent ion and monitoring the collision-induced product ion m/z 452.

**Gpt mutation assay and sequencing analysis.** The liver of each animal was pulverized in liquid nitrogen and divided into aliquots of ~25 mg. Genomic DNA was extracted from 25 mg liver tissue using RecoverEase DNA Isolation Kit (Agilent Technologies, Santa Clara, CA); subsequently, λ–EG10 phages were packaged in vitro from the genomic DNA using Transpack Packaging Extract (Agilent Technologies) following the manufacturer’s instructions. The 6-thioguanine (6-TG) selection assay was performed as previously described (Nohmi et al., 1996). Briefly, Escherichia coli YG6020 expressing Cre recombinase was infected with λ–EG10 phages rescued from murine genomic DNA and incubated on selective media containing either chloramphenicol (CM) at 25 μg/ml or CM (25 μg/ml) plus 6-TG (25 μg/ml) for 72 h until the appearance of colonies. Confirmation of the recovered 6-TG-resistant phenotype was achieved by restreaking mutant colonies on selective media containing CM plus 6-TG. DNA was isolated from confirmed 6-TG-resistant mutants using a Miniprep Kit (Qiagen) according to the manufacturer’s instructions. Sequencing of the gpt gene was performed at the Biopolymers Facility at Harvard Medical School (Boston, MA) using AMPPure beads (Agencourt) and a 3730xl DNA Analyzer (Applied Biosystems) using the forward primer: 5'-TCTCGGCCAACCTATTTCCTCC-3'. Sequences were aligned with the E. coli gpt gene (GenBank M13422.1) using NCBI Nucleotide Blast. Duplicate identical mutations from the same tissue sample were excluded from calculation of mutation frequency (MF) to avoid bias attributable to clonal expansion of sibling mutations. The MF was calculated by first taking the number of confirmed mutations and multiplying by the ratio of independent mutants to the total number of analyzed (sequenced) mutants and then dividing...
this product by the total number of colonies on the Cm only (control) plate. Samples from 33 mice were sequenced. Mutation frequencies and spectra were subjected to statistical analysis.

**Statistical Analysis.** Student’s two-tailed t tests using PRIZM (Graphpad Software, Inc.) were used to determine the significance of differences in DNA adduct levels and mutation frequencies between experimental groups. Mutational spectra were compared using the Adams-Skopek test (Adams and Skopek, 1987; Cariello et al., 1994).

**RESULTS**

**Time Course of AFB₁ DNA Adducts in Neonatal Male and Female Mice**

Newborn gpt delta B6C3F1 mice were administered AFB₁ using two regimens adapted from those previously shown to induce a high incidence of HCC (Fig. 1; Vesselinovitch et al., 1972). A time-course analysis of levels of AFB₁-N²-guanine in liver DNA was first performed in 4-day-old animals after administration of a single 6 mg/kg ip dose of AFB₁. Figure 2 shows the levels of AFB₁-N²-guanine in male and female mice at intervals up to 48 h after dosing. The average levels of AFB₁-N²-guanine were uniformly lower in females than in males, but due to interindividual variation the differences were not statistically significant. The highest levels of AFB₁-N²-guanine in liver DNA of both sexes (male, 9.4 ± 2.9; female 8.3 ± 4.3 adducts/10⁶ nucleotides) were found at the earliest time point (2 h) after dosing. As previously observed in other species, adduct levels rapidly declined between 2 and 10–12 h. A second phase of slower adduct removal occurred between 10 and 48 h, with ~10% of the peak level of AFB₁-N²-guanine attained at 2 h remaining at 48 h. This decline in AFB₁-N²-guanine could have resulted from its conversion to AFB₁-FAPY (the imidazole ring-opened form of AFB₁-N²-guanine), depurination, or enzymatic repair by repair enzymes. These results showed that there were no major gender differences in metabolic activation of AFB₁ by P450s or other biochemical process that determine the fate of the AFB₁-N²-guanine adduct in neonatal liver DNA. Further experiments were designed to examine whether levels of DNA adducts induced by the single- or multiple-dose regimens resulted in a gender difference in initial mutations that tracked with eventual tumor burden. To compare adduct levels, liver DNA was isolated from mice euthanized 24 h after administration of either treatment regimen (Fig. 1) and analyzed for amounts of both AFB₁-N²-guanine and AFB₁-FAPY adducts (Fig. 3). A single dose of 6 mg/kg AFB₁ administered on postnatal day 4 produced similar levels of AFB₁-N²-guanine in males and females as observed at 24 h in the kinetic study described above (Fig. 3A); male 2.7 ± 1; female 2.6 ± 0.8 adducts/10⁶ nucleotides. Levels of the highly mutagenic AFB₁-FAPY were twice as high as those of AFB₁-N²-guanine in both sexes; male 5.5 ± 3 and female 5.8 ± 2 adducts/10⁶ nucleotides.

The multiple dose regimen of 3 × 2 mg/kg AFB₁ given on days 4, 7, and 10 resulted in lower levels of adducts measured 24 h after the final dose compared with the single 6 mg/kg dose. As shown in Figure 3B, the levels of AFB₁-N²-guanine were 6- to 7-fold lower; male 0.36 ± 0.2 adducts/10⁶ nucleotides, female 0.40 ± 0.2 adducts/10⁶ nucleotides. Levels of AFB₁-FAPY adducts were also lower in the multiple dose animals, but only by 1.5- to 2-fold compared with the single dose; male 3.6 ± 2 adducts/10⁶ nucleotides, female 2.5 ± 1 adducts/10⁶ nucleotides. This pattern is consistent with the persistence and accumulation of FAPY adducts during chronic administration of AFB₁ that has been observed in other animal models (Croy and Wogan, 1981). Importantly, however, levels of AFB₁-N²-guanine or AFB₁-FAPY induced by either treatment protocol showed no evidence of gender-related differences.

**Mutation Frequencies in the gpt Gene of AFB₁-Treated Neonatal Mice**

The early-stage genotoxicity and mutagenesis of AFB₁ adducts in liver DNA were assessed in the gpt transgene following selection for 6-TG resistance. A minimum of four mice of each sex were treated with the single- or multiple-dose regimens as described above. Based on previous studies (Thybaud et al., 2003), for mutation analysis, livers were collected from animals euthanized 21 days after the final dosing. Figure 4 shows significant increases in MF in livers of mice treated with AFB₁ administered by either the single- or multiple-dose regimens. In DMSO-treated mice, the average MF was 3 ± 1, which is approximately half the spontaneous MF reported for gpt delta C57BL/6J mice (Masumura et al., 1999). The MF induced by the single-dose AFB₁ regimen in males and females combined was 92 ± 14 × 10⁻⁶. A lower MF was observed in mice treated with the multiple-dose regimen; 66 ± 19 × 10⁻⁶ (p = 0.01, single vs. multiple dose).

FIG. 1. Experimental scheme for treatment of newborn gpt delta B6C3F1 mice with AFB₁, analysis of AFB₁-DNA adducts and mutations in the gpt gene in liver.

FIG. 2. Amounts of AFB₁-N²-guanine in liver DNA of 4-day old male (– -) and female (– -) gpt delta B6C3F1 mice after administration of 6 mg/kg AFB₁. Points = mean ± SD.

FIG. 3. AFB₁ DNA adducts in liver of gpt delta B6C3F1 mice 24 h after a single 6 mg/kg dose (A) or 3 × 2 mg/kg administered every third day (B). Plotted data = mean ± SD.
This is consonant with the lower amounts of mutagenic AFB1 adducts found at 24 h following the multiple dose protocol. There was no significant difference in the mutation frequencies observed in males as compared with females treated with either regimen, indicating that liver cells of both sexes were similarly susceptible to mutagenesis by AFB1.

**Mutation Types in the gpt Gene**

DNA sequence analyses were performed on 6-TG-resistant mutants isolated from AFB1- and DMSO-treated mice. Although fewer 6-TG-resistant mutants from the DMSO-treated controls than from AFB1-treated mice were available for sequencing, the types of mutations present in both were similar. Tables 1 and 2 summarize the types of mutations in livers of males and females treated with the two regimens. A total of 264 6-TG-resistant colonies from 17 AFB1-treated mice were analyzed by DNA sequencing to identify mutations present in the 459 bp gpt gene. Mutations found included base substitutions (248/264, 94%), insertions (4/264, 2%), and small deletions (12/264, 5%). Both transitions and transversions were present at G:C and A:T base pairs. The most frequent type of mutation seen in all the AFB1-treated groups was the G:C to T:A transversion (169/264, 64%) followed by G:C to A:T transition (44/264, 17%). Single base pair deletions occurred primarily at G:C base pairs (91/11, 81%). Overall, the types of mutations were similar in male and female mice treated with either a single dose (6 mg/kg) or multiple doses (3 × 2 mg/kg) of AFB1.

Sequence analysis of 62 mutants obtained from 16 DMSO-treated mice (combined data from both the single- and multiple-dose groups) revealed that G:C to A:T transversions were the predominant mutation (22/62, 35%) followed by G:C to T:A transversions (13/62, 21%). Single base pair deletions at G:C base pairs were found at the same frequency as in AFB1-treated animals (79, 78%). As described below, distribution of these mutations within the gpt gene differs significantly from that of AFB1-treated mice.

**Comparison of Mutational Spectra**

Differences in the distribution of mutations with regard to base number in the gpt gene among treatment groups were tested using Monte Carlo analysis (Adams and Skopek, 1987). Mutation spectra were significantly different between AFB1-treated groups, there was no significant difference in the types or positions of mutations (multiple dose vs. single dose, P = 0.229). Thus, the AFB1 mutational spectra in the gpt sequence were not dependent on the exposure conditions in this experimental model. Further comparisons of mutational spectra between male and female animals showed that they were also indistinguishable.

**DISCUSSION**

This study examined relationships among levels of liver AFB1-DNA adducts and mutagenesis in liver cells of newborn transgenic gpt delta B6C3F1 mice.
mice treated with dosing protocols previously shown to be highly effective in inducing HCC in male, but not female, B6C3F1 mice (Vesselinovitch et al., 1972). We found that dosing with AFB\(_1\) created very similar levels of adducts in liver DNA of both sexes and induced nearly identical mutation frequencies and types of mutations in the gpt transgene in liver cells. These results demonstrate that during infancy, male and female B6C3F1 mice are at similar risks of genotoxic damage and mutagenesis resulting from AFB\(_1\) exposure. Monitoring of AFB1-DNA adducts revealed that administration of a single 6 mg/kg dose produced a higher level of DNA adducts at 24 h compared with the multiple dose regimen. Levels of AFB1-N7-guanine decreased rapidly during the 24 h after treatment, which has been shown to be a result of its removal by DNA repair processes together with its chemical transformation into secondary lesions, including apurinic sites and two imidazole ring-opened AFB1-FAPY forms that are believed to be rotamers (Brown et al., 2006). The lower level of adducts in liver DNA induced by treatment with the multiple-dose regimen was reflected in a lower gpt MF observed in animals treated with multiple doses of AFB\(_1\). Our results identified G:C to T:A transversions in the gpt gene as the most prevalent mutation induced by AFB\(_1\) in the liver of mice. Studies of similar design by other investigators produced similar findings, with AFB\(_1\) inducing primarily G:C to T:A (76%) mutations in the cII gene in the neonatal liver of Big Blue mice (Chen et al., 2010). Overall, the types of mutations found in the gpt gene of AFB\(_1\)-treated mice were characteristic of those induced by it in other experimental systems. The G:C to T:A transversion is the predominant mutation induced by either the AFB\(_1\)-N\(^7\)-guanine or AFB\(_1\)-FAPY adduct in in vitro experimental systems (Bailey et al., 1996; Foster et al., 1983; Smela et al., 2002). In E. coli, the AFB\(_1\)-FAPY minor rotamer was found to be the most potent mutagen, producing G:C to T:A transversions about six times more frequently than AFB\(_1\)-N\(^7\)-guanine (Smela et al., 2002). The high mutagenic potency of the FAPY adduct together with its persistence in DNA suggests that it could be responsible for a major fraction of the mutations induced by AFB\(_1\) in the rapidly growing neonatal liver.

Several mutational hot spots for G:C to T:A transversions in the gpt gene were observed in livers of AFB\(_1\)-treated mice, including nucleotide positions

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**FIG. 5.** Nucleotide sequence of the gpt gene indicating the position and type of base substitutions, deletions (□) and insertions (V) induced by a single 6 mg/kg AFB\(_1\) dose administered to 4-day-old gpt delta B6C3F1 mice. Mutations were assayed 21 days after dosing.
101, 108, 115, 140, 208, 244, and 320. Such hot spots may result from structural features of the DNA sequence that increase the reactivity of guanine with the AFB$_1$-8,9-oxide (Benasutti et al., 1988), actions of the repair and replication processes, or properties of the gpt gene product used to select 6-TG-resistant mutants. The selection procedure requires nearly complete inactivation of the _E. coli_ gpt enzyme since toxic levels of 6-TG remain even in the presence of very low enzyme activity (Thilly et al., 1978). In this regard, it is noteworthy that the most frequently encountered hot spots, including positions 108, 115, 140, and 208 are located in codons for amino acids involved in the binding sites of guanine or phosphoribosylpyrophosphate, the enzyme’s two substrates (Vos et al., 1998). This observation provides a plausible explanation of the partial overlap observed between AFB$_1$-induced mutational spectra and those of other agents. Furthermore, it suggests the possibility that unrecognized mutagenic hot spots may exist at nucleotides in the _gpt_ sequence that result in amino acid changes that do not strongly affect gpt enzyme activity. If this were the case, MF values calculated from our data would represent minimal estimates.

In both experimental animals and humans, the incidence of HCC is much higher in males than in females. Our findings of similar levels of AFB$_1$ adducts and mutation frequencies in infants of both sexes suggests the possibility that biochemical or other factors may be responsible for the observed sex difference in HCC incidence occurring in adult mice treated with AFB$_1$ as infants. Current conceptual models of HCC pathogenesis are based on initiating mutations produced by genotoxic compounds followed by enhanced tumor development driven by factors such as liver cell regeneration and chronic inflammation. In humans, aflatoxin synergizes with hepatitis B virus (HBV) to greatly increase the risk for HCC (Wild and Montesano, 2009). Host responses to HBV infection include activation of the nuclear factor kappa-B signaling pathway that contributes to chronic inflammation and promotion of HCC (Sun and Karin, 2008). One explanation offered for the resistance of female mice to HCC is the observation that estrogenic hormones can suppress inflammation and reduce cancer risk during the promotion phase of hepatocarcinogenesis in mice (Nakatani et al., 2001; Naugler et al., 2007). Although mechanisms responsible for different sex-based incidence of HCC remain to be defined, our findings support the suggestion that postinitiation host responses are important modulators of HCC development.

Aflatoxin-DNA adducts are validated biomarkers of AFB$_1$ exposure and have enabled estimation of risk for HCC in exposed human populations (Groopman et al., 2008). In experimental animals, the chemoprotective agent oltapraz has been shown to be effective in inhibiting formation of AFB$_1$-DNA adducts and reducing the mutagenicity of aflatoxin B$_1$-adducted DNA. In vitro and in vivo studies indicate that oltapraz reduces the formation of aflatoxin B$_1$-DNA adducts and reduces the risk for HCC in exposed human populations (Groopman et al., 2008). In experimental animals, the chemoprotective agent oltapraz has been shown to be effective in inhibiting formation of AFB$_1$-DNA adducts and reducing the mutagenicity of aflatoxin B$_1$-adducted DNA. In vitro and in vivo studies indicate that oltapraz reduces the formation of aflatoxin B$_1$-DNA adducts and reduces the risk for HCC in exposed human populations (Groopman et al., 2008).

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/

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