Effect of *Escherichia Coli* Endotoxin on the Developing Rat Liver. I. Giant Cell Induction and Disruption in Protein Metabolism

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Summary

In an attempt to determine the effect of a toxic insult occurring *in utero* on the developing liver, fetal amniotic cavities of timed-pregnant (16-day) Sprague-Dawley rats were injected with varying concentrations of *E. coli* endotoxin. Controls were sham-operated or saline-injected fetal rats. Five days after injection, the pregnant animals were killed; ^{14}C -leucine was injected subcutaneously into the newborns, and their livers were removed, prepared for histologic examination and for analysis of radiolabeled protein incorporation. A significant increase in the number of hepatic giant cells (giant cells/mm²) was noted in fetal rats injected with endotoxin compared to the controls ($P < 0.01$). In addition, the incorporation of ^{14}C -leucine into total liver protein and immunoprecipitable albumin of endotoxin-injected fetuses was significantly lower than that of control animals ($P < 0.01$ and $P < 0.02$, respectively). In contrast, ^{14}C -leucine incorporation into immunoprecipitable α -fetoprotein was significantly greater in endotoxin-injected fetuses than in controls ($P < 0.02$). Also, serum α -fetoprotein levels were significantly ($P < 0.01$) higher in the endotoxin-injected fetuses.

These data suggest that intrauterine insult with endotoxin during late fetal development results in hepatotoxicity manifested as giant cell induction and disrupted protein metabolism. The changes (hepatic giant cell response and elevated serum levels of α -fetoprotein) in rat liver parallel those reported in human newborns with neonatal hepatitis syndrome. Therefore, it may be suggested that the endotoxin-injected fetal rat may represent an appropriate animal model for the study of the pathogenesis of neonatal hepatitis in man.

Speculation

The development of an animal model for neonatal hepatitis syndrome may ultimately provide the basis for a better understanding of the pathogenetic mechanisms responsible for both morphologic and biochemical abnormalities noted in this spectrum of hepatobiliary diseases. Of particular importance is the more precise characterization of the hepatic giant cell frequently demonstrated in neonatal hepatitis. This cell type could contribute to the pathogenesis of hepatitis by releasing secretions that help to regulate changes in the hepatic cellular environment such as proteolysis, inflammatory cell infiltration, or disruption of the biliary tract epithelial surface, and thereby, contribute to the various manifestations of disease. Furthermore, there could be modification of these tissue reactions by coexisting genetic factors such as renewed synthesis of fetoprotein after reversion of fetal hepatocytes to an earlier stage of development. The synthesis and release of this developmental protein may also be of importance in immunoregulation during intrauterine existence.

The pathogenesis of neonatal hepatitis syndrome is obscure. Evidence is still lacking that a toxic intrauterine or neonatal insult

contributes to the development of the histopathologic abnormalities that occur in this condition. The characteristic hepatic giant cell change, regularly associated with neonatal hepatitis, may be a stress response resulting from environmental factors that interfere with intrauterine development. Alternately, this response may occur because of environmental interference with growth and differentiation in early postnatal life. The appearance in the liver of large multinucleated cells resembling the giant cells of human neonatal hepatitis have been reported in animal studies after exposure to a variety of hepatotoxic agents including carbon tetrachloride (17), colchicine (12), rat virus (11), and *E. coli* endotoxin (3). However, attempts to study fetal hepatotoxicity by ip injection of pregnant animals have resulted in confusing and inconsistent histologic abnormalities of neonatal liver including sporadic giant cell induction. This study was, therefore, designed to attempt to establish a consistent response by the developing liver to *direct E. coli* endotoxin (endotoxin) injection into fetal amniotic cavities at a gestational stage when morphologic and functional features of the liver are well developed (5). Endotoxin, a known biologic toxin, was selected because of previous reports of morphologic evidence for hepatotoxicity (3, 9) in experimental animals, similar to the giant cell change noted in neonatal hepatitis syndrome.

MATERIALS AND METHODS

Timed-pregnant (16-day) Sprague-Dawley rats were obtained from Charles River Breeding Labs (Wilmington, MA). Animals were housed in individual cages and allowed *ad lib* access to water and rat chow (Ralston Purina Co., Minneapolis, MN). Under ether anesthesia, the pregnant rats were subjected to laparotomy and the gravid uterine horns were exposed. Endotoxin (Bactolipopolysaccharide *E. coli* 055:BS, Difco Laboratories, Detroit, MI) was then injected into the fetal amniotic sacs (4) in concentrations ranging from 0.0003–3.0 $\mu\text{g}/\text{ml}$ with 30 μl as the standard injection dose. Control fetuses were either nonperturbed, sham-operated, or given intraamniotic injections of 30 μl normal saline. The abdominal cavity of the pregnant animal was then closed; 5 days later, the fetuses were surgically removed in order to assess survival, obtain liver for histologic sections and protein studies, as well as to obtain serum for total protein, albumin, and α -fetoprotein (AFP) concentrations. Dead fetuses or fetuses more than 0.5 g below the mean fetal weight of the saline-injected group were excluded from the study.

After removal of the rat fetuses, they were weighed and killed by decapitation. Fetal livers were dissected and representative tissue fixed in Bouin's solution for staining with hematoxylin and eosin. Liver sections were coded and examined without knowledge of source. Sections were examined under the light microscope for giant cell formation and for inflammatory cell infiltration. Giant cells were arbitrarily defined by two criteria: 1) cytomegaly of at least three times average liver cell size and 2) multinucleation, or

the presence of at least three nuclei (13). Using a calibrated lens (grid square measured to be 0.5 mm² at 160 X, Zeiss Inc., Frankfurt, Germany), cells were counted in 10 separate fields representing a total area of 2.5 mm² (10 × 0.5 × 0.5) and the number of giant cells expressed/mm².

Fetal serum AFP and albumin concentrations in endotoxin-injected and control animals were measured by electroimmunodiffusion (7) in 1% agarose containing sodium barbital buffer (pH 8.6) and rabbit AFP or albumin antibody (concentrations of 3 and 0.46%, respectively). Rabbit antirat AFP (and AFP standard) was kindly provided by Elliot Alpert, M. D. (Massachusetts General Hospital, Boston, MA). Rabbit antirat albumin was prepared in this laboratory using techniques previously described (18). Both antisera were determined to be monospecific by Ouchterlony immunodiffusion and counter immunoelectrophoresis against normal and fetal sera, and amniotic fluid. Standards for AFP and albumin were prepared from homogenized 15th day of gestation fetal rats with amniotic fluid and purified rat albumin (Sigma Chemical Company, St. Louis, MO), respectively. Total protein concentrations were also determined by the method of Lowry *et al.* (10).

Livers from endotoxin-exposed (16th day of gestation) rats were isolated by dissection 45 min after parturition and subcutaneous injection of ¹⁴C-leucine (1μCi/g body wt, New England Nuclear, Boston, MA; specific activity = 54.5 μCi/mmol). Liver specimens were carefully weighed and homogenized in 10 volumes of phosphate buffered saline. An aliquot of the homogenate was precipitated for protein determinations using equal volumes of 20% trichloroacetic acid (TCA). Protein precipitates were washed three times with ice cold 10% TCA and then dissolved in 1 ml of NaOH (1N). Ten microliters of resuspended TCA precipitate and 500 μl of the supernatant were mixed with 10 ml of Aquasol (Beckman LS-250, Anaheim, CA), and radioactivity determined by liquid scintillation spectrometry. TCA-precipitated protein was determined by the method of Lowry *et al.* (10). ¹⁴C-leucine incorporation into total protein was expressed as dpm/mg protein.

¹⁴C-leucine incorporation into albumin and AFP by fetal liver specimens was determined by modification of a previously described technique utilizing solid-phase immunoprecipitation (15). The immunoprecipitation reaction was carried out at equivalence for albumin and AFP with addition of unlabeled albumin carrier and no added AFP carrier, respectively. The reaction was incubated at 37°C for 1 hr and then at 4°C for an additional 18 hr. To minimize nonspecific radioactive contamination, the immunoprecipitate containing either, ¹⁴C-albumin or ¹⁴C-AFP was centrifuged at 10 × g for 10 min in media containing 1 M sucrose-1% Triton mixture. The precipitated antigen-antibody complexes, containing ¹⁴C-radiolabeled albumin or AFP were separated from the sucrose-Triton cushion by removal of the distal portion of the centrifuge tube. After rapidly freezing the precipitate, radiolabeled albumin and AFP activity were determined in a liquid scintillation spectrometer.

Incorporation of ¹⁴C-leucine into albumin and AFP was expressed as percent of radioactive incorporation into total protein (e.g., dpm_{albumin}/dpm_{protein} × 100). In these experiments, the specific activity (dpm/mg protein) of liver proteins was assumed to reflect protein synthesis because the specific activity of the fetal liver-free leucine pool, fetal liver weights, and fetal body weights were approximately the same in all treatment groups. Amino acid analysis was performed with a D-500 analyzer (Durrum Instruments Corporation, Palo Alto, CA).

Statistics: All determinations of statistical significance were made by utilizing Student's *t* test.

RESULTS

Figures 1a and 1b illustrate representative histologic sections of 21-day fetal rat liver after exposure to endotoxin on the 16th day of gestation. Numerous hepatic giant cells appeared after this hepatotoxin exposure; these large cells had at least three nuclei and in some instances as many as 20 nuclei. Furthermore, their bulky cytoplasm was pale and contained fine basophilic granules.

Fig. 1. Histologic sections of 21-day fetal liver after exposure to intraamniotic endotoxin showing *a* numerous giant multinucleated cells, (hematoxylin and eosin stain; magnification × 160) and *b* a single giant multinucleated cell (arrow) near a sinusoid (S) at higher power, (hematoxylin and eosin stain; magnification × 400).

Intracellular bile, bile canaliculi, and larger bile ducts were not observed. Numerous hematopoietic cells were present in the sinusoids which were lined by hyperplastic Kupffer cells, and there was mild round cell infiltration of the liver parenchyma. Fetal hepatocytes showed loss of cellular outline and were markedly vacuolated and also devoid of intracellular bile.

A correlation was noted between the dose of endotoxin injected and fetal rat survival as well as liver giant cell induction (Fig. 2). A striking increase in cellular response (giant cells/mm²) was noted at an endotoxin concentration of 0.03 μg/ml; however, the response plateaued beyond this level of endotoxin injection. Above this same concentration (0.03 μg/ml), a precipitous decrease occurred in percent fetal survival. Therefore, it was necessary to confine the quantitative radiolabeling experiments to this endotoxin level. There was a significantly greater number of giant cells in livers from fetal rats exposed to endotoxin compared to controls (*P* < 0.01) except at the lowest endotoxin concentration (Fig. 3). However, a small number of giant cells were observed in control fetal livers, which may be due to the effect of endogenous endotoxin or to ether anesthesia exposure. There was no significant difference in the number of giant cells in livers obtained from the various control (nonperturbed, sham-operated, and saline-injected) fetal rats. Furthermore, 100% fetal rat survival was observed for all groups of control animals.

Serum levels of protein also correlated with the amount of endotoxin injected into fetal cavities. A precipitous decrease in serum total protein was noted at increased endotoxin concentrations. However, while serum AFP levels in endotoxin-injected

Fig. 2. Effects of *E. coli* endotoxin on giant cell induction and fetal rat survival. Note dose-related ($\mu\text{g}/30\mu\text{l}$ as the standard injection volume) changes in giant cells/mm² and % survival. Each point with vertical lines represents the mean $\pm$ SE for giant cells/mm² ($n = 10-14$ rats).

Fig. 3. Mean $\pm$ SE numbers of giant cells/mm² after exposure to varying concentrations ($\mu\text{g}/30\mu\text{l}$) of intraamniotic *E. coli* endotoxin ($n = 10-14$ rats). The stippled bar represents the mean $\pm$ SE for all controls ($n = 28$ rats). Toxin, *E. coli* endotoxin.

fetuses were significantly greater than in controls ($P < 0.01$) except at the highest endotoxin concentration, serum albumin levels in endotoxin-injected animals were not different than controls (Fig. 4).

The mean $\pm$ SE value for incorporation of ¹⁴C-leucine into total protein by liver in control rats was $24,370 \pm 2990$ dpm/mg protein (Fig. 5). In contrast, the mean incorporation values for endotoxin-injected ($0.03 \mu\text{g}/\text{ml}$) rats was $11,676 \pm 265$ dpm/mg protein. This level of incorporation was significantly less than that of control levels ($P < 0.01$). In like manner, the incorporation of leucine into liver albumin, expressed as a percent of total protein incorporation, was 7.12 ± 1.18 for normal control fetal rats compared to the significantly lower percent incorporation of 1.35 ± 0.59 for endotoxin-injected rats ($P < 0.02$). Finally, incorporation of leucine into liver AFP was 6.87 ± 1.55 for normal control fetal rats compared to the significantly higher percent incorporation of 12.68 ± 2.60 ($P < 0.02$) for endotoxin-injected rats (Fig. 5).

Fig. 4. Mean $\pm$ SE levels of serum total protein, albumin, and α -fetoprotein after varying concentrations ($\mu\text{g}/30\mu\text{l}$) of intraamniotic *E. coli* endotoxin ($n = 10-14$ rats). α FP, alpha-fetoprotein.

Fig. 5. Effect of *E. coli* endotoxin ($0.03 \mu\text{g}/\text{ml}$), injected into amniotic cavities of fetal rats, on radiolabeled leucine incorporation into liver total protein, albumin, and α -fetoprotein. Vertical lines denote $\pm$ SE ($n = 10-14$ rats). % leucine incorporation refers to lined and stippled bars in both panels on the right, separated by the dotted line. α FP, alpha-fetoprotein.

DISCUSSION

In this study, using endotoxin as an intrauterine toxin for the developing rat liver, a significant and consistent abnormality in liver histology and in protein metabolism has been demonstrated. The results suggest that giant cell change may be associated with endotoxin exposure to rat fetal hepatocytes. These large multinucleated cells could represent reparative, fetal hepatocyte proliferation which has been associated with increased serum levels of AFP (1). It has been shown by electron microscopy that these poorly defined cells possess a prominent rough endoplasmic reticulum (16) and therefore have the potential for synthesis of protein. They may be partly responsible for the elevated levels of serum AFP in the fetal rats injected with the highest endotoxin concentrations and demonstrating the maximum hepatic giant cell induction. In addition, the significantly increased percent leucine incorporation into AFP for the endotoxin-injected rats compared to the control rats suggests accelerated synthesis of AFP by the perturbed fetal rat livers. Interestingly, the biosynthesis and release of AFP *in vitro* is related to the actively proliferating cell (8). Hence, if the appearance of giant cells is secondary to a reparative, proliferative process, they may synthesize fetoprotein; however,

they have not as yet been characterized as proliferating, DNA synthesizing cells by the standard techniques of thymidine incorporation or autoradiography. An alternative explanation for accelerated production of AFP after endotoxin hepatic damage may be reversion of fetal hepatocytes to a more primitive, but functional developmental stage, perhaps representing alteration of the genetic control of this protein.

In the present study, serum AFP levels were significantly higher in endotoxin-injected fetuses than control fetuses in all instances except at the highest level of endotoxin. At this concentration of endotoxin (3 µg/ml) the failure to cause increased serum AFP concentrations may reflect the lower survival of fetal hepatocytes and a concomitant decrease in reparative hepatocyte proliferation. The precipitous decrease in the serum total protein at increased endotoxin concentrations also reflects the extent of hepatocellular damage and is consistent with the poor fetal rat survival observed at the higher endotoxin concentration. For short-term experiments, however, serum albumin levels in endotoxin-injected animals were not appreciably different from controls; this may be due to the extended half-life of albumin which prohibits rapid changes in serum levels to occur with acute liver injury. Yet, radiolabeled leucine incorporation into albumin, a reflection of acute change in hepatic albumin synthesis, was significantly depressed in the fetal rat livers exposed to endotoxin suggesting a decrease in albumin synthesis. Furthermore, radiolabeled leucine incorporation into AFP was significantly increased in the endotoxin exposed rat livers suggesting an increase in AFP synthesis. In normal development, albumin synthesis is inversely related to AFP synthesis and increases with advancing gestational age (14).

In summary, the injection of endotoxin into the amniotic cavity results in a reproducible fetal hepatotoxic event consisting of the induction of giant cells and significant alteration of AFP, albumin, and total protein metabolism. The direct intraamniotic route of injection may be more effective than maternal ip injection during pregnancy because of inactivation of the toxin by maternal liver. It is suggested that endotoxin exposure of fetal rats may be a useful model for study of the pathogenesis of the neonatal hepatitis syndrome because a hepatic giant cell response (6) and elevated serum levels of AFP (2, 19) consistently occur in this condition.

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