The effects of acute and chronic prenatal alcohol exposure on lymphoid organ development and immune function in C57BL/6 mice
by Ching Huang

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Fetal alcohol syndrome (FAS) and immune alteration have been linked in humans and documented in experimental animal models. A fully developed immune system plays an important role in immune function throughout life. Unfortunately, there are relatively few reports systematically examining the consequences of prenatal alcohol exposure to fetal immune organ development and longer term immune function capability.

In this study, I attempted to determine the effects of alcohol exposure in utero on C57BL/6 mice through acute (intraperitoneal injection) and chronic (23% ethanol derived diet feeding) administration methods to fill this gap. The results showed that following acute ethanol exposure, 18 day old fetuses displayed classical features of FAS including missing digits, low body weight, resorption, and significantly lower number of fetal thymocytes.

On the other hand, 4-8 week-old young adults chronically exposed to ethanol during prenatal life showed no significant changes in immune function, as assessed by a battery of immunological tests. Tests of cellular immunity included delayed-type hypersensitivity, contact sensitivity, cytotoxicity, allogeneic mixed lymphocyte reaction, syngeneic mixed lymphocyte reaction and RCS driven proliferation of Con-A blasts. Humoral responses were analyzed by determining plaque forming cells for either T-dependent or T-independent antigens.

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ON LYMPHOID ORGAN DEVELOPMENT AND IMMUNE FUNCTION

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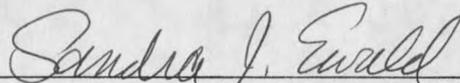Bozeman, Montana

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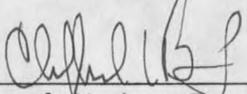
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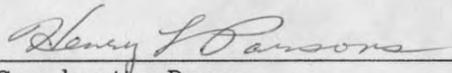
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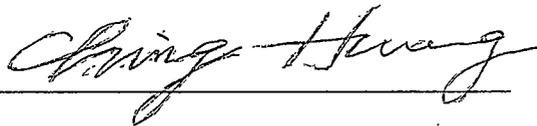
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ABSTRACT

Fetal alcohol syndrome (FAS) and immune alteration have been linked in humans and documented in experimental animal models. A fully developed immune system plays an important role in immune function throughout life. Unfortunately, there are relatively few reports systematically examining the consequences of prenatal alcohol exposure to fetal immune organ development and longer term immune function capability.

In this study, I attempted to determine the effects of alcohol exposure in utero on C57BL/6 mice through acute (intraperitoneal injection) and chronic (23% ethanol derived diet feeding) administration methods to fill this gap. The results showed that following acute ethanol exposure, 18 day old fetuses displayed classical features of FAS including missing digits, low body weight, resorption, and significantly lower number of fetal thymocytes.

On the other hand, 4-8 week-old young adults chronically exposed to ethanol during prenatal life showed no significant changes in immune function, as assessed by a battery of immunological tests. Tests of cellular immunity included delayed-type hypersensitivity, contact sensitivity, cytotoxicity, allogeneic mixed lymphocyte reaction, syngeneic mixed lymphocyte reaction and RCS driven proliferation of Con-A blasts. Humoral responses were analyzed by determining plaque forming cells for either T-dependent or T-independent antigens.

INTRODUCTION

Alcohol-related health problems are among the most
serious public health problems around the world (21). Alcohol
has been associated with a number of human illnesses,
including central nervous system (CNS) depression, physical
dependence, nutrient deficiency, cardiomyopathy, liver
cirrhosis, gastrointestinal tract problems, pancreas damage,
and hematologic and renal defects (22). It also increases the
risk of cancer development (22) and has long been suspected as
a teratogenic agent. According to a Gallup Poll, the
percentage of women in the United States who drink alcohol
increased from 45% to 66% over the last forty years (10).
With the increasing alcohol consumption by pregnant women, a
pattern of birth malformations and fetal growth retardation of
infants borne by alcoholic mothers was identified and named
the Fetal Alcohol Syndrome (FAS) in 1973 by Jones and Smith
(46,47). Since then, there has been increasing evidence of a
causal relationship between alcohol consumption by pregnant
females and occurrence of FAS. In 1984, it was estimated that
there were about 6,550 to 11,000 children born each year in
the United States with major or minor physical birth defects
caused by prenatal alcohol exposure (77).

FAS has been characterized by diagnosis of infants with
signs in some of the following categories (18,19,34,42,67,81):

1. Prenatal and/or postnatal growth retardation: low birth weight, small head size.

2. Central nervous system dysfunction: signs of neurologic abnormality, intellectual impairment, mental retardation.

3. Specific cluster of facial abnormalities: microcephaly, short palpebral tissues, poorly developed philtrum and thin upper lip.

4. Limb anomalies: joint anomalies, cutaneous, skeletal and muscular malformation.

5. Cardiac defects: ventricular septal defects.

6. Development delay: external genital maturation delay.

FAS as well as alcohol problems in adults have been examined from many different aspects such as embryology (48), teratology (48), endocrinology (6,43), biochemistry (60), and clinical and basic neuro-immunology (24,44). The effects of prenatal alcohol exposure on human embryo development were multiple. Since 1973, when FAS was coined by Jones and Smith (46,47), there have been more than 1,000 clinical and experimental reports about the impact of prenatal alcohol exposure on fetal development (see for example 40,51,20,85,84, 83,79,31,32,70). Moreover, laboratory animal models of FAS have been developed in mouse (11,17,71,73), rat (8,9,13,16), and monkey (65), and many of the features of human FAS were seen in such animal models.

In spite of many reported cases of human FAS and studies of animal models of FAS, many questions about the effects of

prenatal alcohol exposure on fetal development remain unanswered. For example, little is known about the effects of prenatal alcohol exposure on the development of lymphoid organs and immune function, which is the subject of this thesis. Chronic exposure to ethanol has been associated with several immune defects of adult humans, including depressed granulocyte chemotaxis (33,55,82), depressed natural killer cell activity (15), and defective mitogen-induced T lymphocyte and B lymphocyte proliferation (33,49). Exposure of adult animals to ethanol caused depression of natural killer cell activity (1,2,58), depression of mitogen-driven proliferation of B lymphocytes and T lymphocytes (4,38,75), inhibition of cell-mediated cytotoxicity (88), and depression of cutaneous hypersensitivity and antibody production (7,87). In addition, adult animals exposed to ethanol had thymic and splenic atrophy (87).

There is little information about the effects of fetal alcohol exposure of humans or animals on lymphoid and immune system development. In 1981, Johnson et al. (45) reported their studies of 13 humans diagnosed as FAS among which there was an increased incidence of bacterial infections, including pneumonia, meningitis, sepsis, otitis media and minor infections. They did a comprehensive immunologic evaluation of cellular and humoral immune responses and showed that the FAS patients had decreased numbers of E-rosette forming T lymphocytes and EAC-rosette forming B lymphocytes as well as

diminished Con-A and pokeweed mitogen-induced proliferative responses. Some FAS children had marked eosinophilia. In these patients, delayed-type hypersensitivity responses, neutrophil counts, total hemolytic complement and absolute lymphocyte counts were not depressed compared with age-matched children of a control group. Johnson et al. suggested that impairment of some immune responses may account for increased susceptibility to infection in FAS children. In 1982, Amman et al. (5) pointed out that four patients with clinical and laboratory features of the DiGeorge Syndrome (congenital absence or hypoplasia of thymus) had a definite history of maternal alcoholism. These four patients showed not only certain clinical abnormalities of DiGeorge syndrome but also of FAS, such as abnormalities of the eyes, ears, mouth, face, cardiomascular system, CNS and immune system; thus, certain symptoms exist in both FAS and DiGeorge syndrome children, and it was reasonable to ask whether fetal alcohol exposure would cause a syndrome, similar to DiGeorge syndrome, of thymus hypoplasia or aplasia followed by immune alteration. The answer to this question is best sought through well-controlled studies of animals.

When I started this thesis research, only two studies of the effect of fetal alcohol exposure on the immune system of animals had been reported. Monjan and Mandell (64) demonstrated that fetal alcohol exposure of rats could depress mitogen-induced lymphocyte blastogenesis, whereas Zidell et

al. (93), based on limited immune assays, found no immunological effects of prenatal alcohol exposure of mice.

Very recently, several groups have reported results of studies of mice or rats exposed to alcohol prenatally. In these studies, prenatal alcohol exposure depressed the Con A induced proliferative response of rat spleen and thymus cells and reduced thymus weight (74), depressed contact sensitivity and local graft-vs-host responses of mice (36,37), and diminished the proliferative response of rat Con A-blast cells to interleukin 2 (66), when animals were tested as adults. Ewald et al. (25) used a chronic alcohol exposure feeding method, 25% ethanol-derived calories (EDC), to study FAS in C57BL/6J mice. They showed that the thymuses of fetal mice exposed to alcohol prenatally were reduced both in thymocyte number and proliferative response to Con-A plus a source of interleukin 2. In addition, these workers showed that the proportions of L3T4-positive and Lyt-2 positive thymus cells were significantly reduced in alcohol exposed fetuses compared to controls. Moreover, by histologic study it was not possible to delineate the thymic cortex and medulla of ethanol exposed mice as could easily be done using control mice (26).

Because my goal was to study the effect of prenatal alcohol exposure on the development of the thymus and the immune system, it is necessary now to briefly review: (a) the normal development of the thymus and the immune system, and

(b) specifics of administration and importance of timing of prenatal alcohol exposure.

The immune system consists of primary and secondary lymphoid organs. Primary lymphoid organs are the thymus, which produces T lymphocytes that are in charge of cellular immune reactions, and bone marrow or fetal liver which produces B lymphocytes that mediate humoral immune reactions. Secondary lymphoid structures include lymph nodes, spleen, tonsils, Peyer's patches, and other lymphoid tissues.

In mouse, the fetal thymus is formed by a non-lymphoid epithelial stroma (derived from the third and fourth pharyngeal pouches) associated with macrophages and dendritic cells and precursor thymocytes that migrate from the yolk sac and form the lymphoid portion of the thymus (3). At day 10 of ontogeny, the epithelial structure (stroma) is constructed and precursors of lymphoid cells (pro-thymocytes) migrate to the thymus anlage at gestational day (g. d.) 10 to g. d. 11 (3). Subsequently the pro-thymocytes proliferate and differentiate into several subpopulations of immature and mature T cells with different surface antigens, such as Thy-1, T cell receptor, L3T4, Lyt 2, Ly-1, and CD3. At the last day before birth (day 19), the thymus appears completely developed and the proportion of different cell types is similar to that found in the adult thymus (3).

In the adult mouse thymus, most thymocytes (about 80%) are both CD4 positive and CD8 positive (in the mouse CD4

equals L3T4 and CD8 equals Lyt-2, respectively). The double-positive T cells are nonfunctional lymphocytes and most of these cells are destined to die inside the thymus although some of them are precursors of single-positive T cells which may migrate to secondary lymphoid organs (30). The rest of the thymocytes are immature double-negative or mature single-positive cells. There are about 8% of CD4-positive CD8-negative cells (primarily helper and inducer T cell subpopulations) and 6% of CD4-negative CD8-positive cells (mostly cytotoxic and suppressor T cell subpopulation). These single-positive cells are the functional, T cell receptor-bearing, mature T cells that are in charge of main immune response of secondary lymphoid tissues.

Pre-B cells appear in fetal liver at day 12 of gestation then continue to the B-cell lineage development through expression of surface antigens such as Ia (MHC antigen) and membrane immunoglobulin (Ig) (at gestational day 16 or 17 in the fetal liver) (50). Pre-B cells also develop into mature, functional B cells in bone marrow. In the B lymphocyte component, there are different structures in birds and mammals for B cell generation. The Bursa of Fabricius, a modified piece of intestine with a connection toward the lumen, is found in birds (68). Mammals including humans do not have the Bursa of Fabricius but instead produce B lymphocytes in fetal liver and bone marrow. The mature B lymphocytes generated from these bursa and bursa equivalent structures are in charge of

humoral immune responses with cooperation with T lymphocytes and lymphokines. The generation of both mature B and T lymphocytes in primary lymphoid organs is followed by their migration to secondary peripheral lymphoid organs.

On the point of thymus maturation in human and mouse, there is some difference. The human thymus matures at the fifteenth week of the forty week gestation period (54), but the mouse thymus matures (day 19) just before birth. Therefore, the mouse thymus is immature during most of the prenatal alcohol exposure period whereas the human thymus is mature during much of the exposure time.

Because the mouse thymus is immature during most of fetal development, it is likely that prenatal alcohol exposure will have different effects if given at different times during fetal development. Daft et al. (23), in 1986, reported heart defects following acute alcohol exposure (two doses, four hours apart, of 2.9g ethanol/kg body weight ethanol exposure through intraperitoneal injection) prenatally to C57BL/6J mouse. They indicated that alcohol exposure of mice at critical times in organ development was associated with abnormal migration of neural crest cells that led to cardiopathology that resembled DiGeorge syndrome (thymus aplasia). Webster et al. (90,91) used the same acute alcohol exposure C57BL/6J mouse model to study FAS. Their results suggested that two doses, four hours apart, of 2.9g/kg body weight ethanol exposure through intraperitoneal injection at

one of the following gestational day 7, 8, 9 and 10 could cause a variety of abnormalities which are seen in children with FAS. Also, particular times of ethanol exposure were associated with specific defects. For example, treatment with alcohol on gestational day 7 or 8 caused a variety of facial abnormalities, whereas treatment with alcohol on gestational day 9 or 10 resulted in limb defects. They also found heart defects on gestational day 8, 9 and 10 of prenatal alcohol exposure but not on gestational day 7.

My goals for this thesis research were: (a) to determine whether prenatal alcohol exposure can alter thymus development of mice resulting in thymic aplasia similar to the DiGeorge syndrome of humans, and (b) to determine whether alcohol-induced fetal thymus alteration of mice can cause changes in immune function after birth.

There were quite a few animal studies showing that acute alcohol exposure resulting in blood alcohol level (BAL) > 400 mg/100ml caused malformation of several organs and systems, but none of these studies focused on the immune system or on thymus development.

In our laboratory, Ewald et al. using a chronic alcohol FAS model showed reduced thymocyte population and size of C57BL/6 mice. Therefore, we chose C57BL/6 as an acute alcohol exposure model. Pregnant mice were injected with 2.9 g ethanol/kg body weight, two doses by intraperitoneal at gestation day 7, 8, 9, 10, 11, plus 12 hour and 16 hour, with

control mice being injected with saline instead. We removed 18-day fetuses and did external physical examinations and thymus cell population analysis.

My second goal was to examine both cellular and humoral immune responses of young adult mice chronically exposed to alcohol in utero. We examined both T and B cell functions in vivo and in vitro of those young adult mice exposed to alcohol prenatally.

The chronic alcohol exposure was by feeding pregnant C57BL/6 mice with 23% ethanol-derived calories (EDC) liquid diet on gestation day 5 to day 19. The liquid diet feeding procedure had been used in our laboratory in previous FAS studies. The advantages of the chronic liquid diet are (a) adequacy of nutrition, (b) no physiological trauma compared to intubation or injection, (c) easy alcohol intake control for most experimental studies of chronic alcohol consumption, (d) alcohol exposure throughout the day instead of only at a certain time during the day. Therefore, the liquid diet feeding technique provides one of the most efficient methods to study the effect of prenatal exposure on immune system function. It also facilitates the comparison with controls by simplifying pair feeding.

Because we studied the long-term (4-8 week after birth) consequences of chronic prenatal alcohol exposure on the immune system development, we used 23% EDC diet instead of 25% EDC in order to get more pups from alcohol-treated mothers.

In our chronic alcohol diet feeding model, we also used foster mothers for both EDC and pair-fed (PF) groups to eliminate the problem that alcoholic mothers may provide poor nourishment, thus assuring that all mice had equal nutrition after birth.

Our results, in acute alcohol exposure experiments, showed no fetal thymus aplasia following prenatal exposure to teratogenic dose of alcohol. We found 18-day fetuses exposed to alcohol on gestational day 9 and 12 hour and 16 hour, had a high incidence of ectrodactyly (missing fourth and/or fifth digits on right forelimb). A high rate of resorption on gestational day 10 or day 11 alcohol treatment was also found. Also, pups from alcohol groups had low fetal weight and lower fetal thymocyte number and fewer cells expressing CD4 and CD8 antigens compared to the saline control group.

In the chronic alcohol exposure experiments, immune function assays of young adult (4-8 weeks) mice showed no difference between EDC and PF group pups in plaque-forming cell assay, delayed type hypersensitivity assay, contact sensitivity response or Con-A blast proliferation. The results of cytotoxicity assays, allogeneic and syngeneic mixed lymphocyte reaction did not show consistent differences.

MATERIALS AND METHODS

Acute Alcohol Exposure

Mice and Mating

C57BL/6J (B6, H-2^b) mice were bred in our Animal Resource Center from stock originally purchased from Jackson Laboratories (Bar Harbor, ME). The mouse room was kept on 12 hr light: 12 hr dark cycle schedule with the dark period being from 1100 to 2300 hr, with temperature of 22 ± 1^0 C. Throughout the experiment, mice were fed with normal chow and water ad-libitum. For mating, adult female mice (2-6 months of age) were housed with one female and one male mouse per cage. The 1 hr mating period was 8 pm to 9 pm, and once a vaginal plug was detected, the pregnant mouse was randomly assigned to either an acute alcohol treatment group or a saline control group.

Acute Alcohol Administration

Using a 1 ml syringe and 27-gauge needle, pregnant C57BL/6J mice were given two intraperitoneal (i.p.) injections of 25% ethanol in saline solution (v/v) of a volume calculated to give 2.9 g ethanol/kg body weight each injection. The two i.p. injections were given 4 hr apart on one gestational day, either day 7 (180 hr and 184 hr after vaginal plug), day 8 (204 hr and 208 hr), day 9 (228 hr and 232 hr), day 10 (252

and 256 hr) or day 11 (276 hr and 280 hr). The control group mice were injected with saline. After injection with saline, both chow and water were removed from the control group mice for 8 hr to match the alcohol treatment group mice condition (after alcohol i.p. injection, mice were intoxicated to a level of sedation or ataxia (57), and thus were not feeding).

Physical Examination of Fetuses

Fetuses from both alcohol treatment and saline control groups were removed at day 18 of gestation. All fetuses were checked for viability, weighed individually and checked for external physical abnormalities before removing the thymus. Then, fetuses were kept in formalin solution for detailed examination, including head size, facial abnormality, and limb defects.

Blood Alcohol Levels (BAL)

For blood alcohol content analysis, we used a different group of nonpregnant mice, because the Metafane anesthetic used before the blood drawing is a teratogen at high concentrations (92). The blood sample was drawn from the retro-orbital sinus 30 min after each i.p. injection. The blood samples were kept in the refrigerator for at least 1 hr to allow clotting to occur. BAL was determined on serum samples by a modified micromethod for measuring alcohol

content using an alcohol dehydrogenase kit from Sigma Chemical
Co., St. Louis, MO (Cat.233-A).

Flow Cytometric Analysis of Thymus Cells

The phosphate minimum essential medium (PMEM) used for
flow cytometric assay cell preparation was made from
Dulbecco's modified Eagle's medium (Irvine Scientific, Santa
Ana, CA; Cat. No. 9418) and supplemented with 0.1% sodium
azide (Sigma Chemical Co. St. Louis, MO), 2% fetal calf serum
(FCS), (Hyclone, Logan,UT), 0.001M Na_2HPO_4, 0.001M $MgCl_2.6$ H_2O
and adjusted to pH 7.0. This medium was sterilized by
filtration through a 0.22 μm membrane.

Thymus cell suspensions of 18-day fetuses were prepared
by teasing the thymus with a 27 gauge needle in PMEM-FCS
medium. For each litter, we pooled three thymus cell samples
together (to get enough cells) according to fetal weight. A
pool contained thymus cells from the largest fetus of a
litter, the smallest fetus of a litter and the fetus closest
to mean weight value of the whole litter. These pooled thymus
cells were counted and adjusted to 2 x 10^7 cells/ml in PMEM.

Flow cytometric analysis was performed by FACS 440
(Becton Dickinson Immunocytometry System, Mountain View, CA)
using an argon laser beam with excitation at 488 nm.
Thymocytes were labeled with three different monoclonal
antibodies: anti-T cell developmental surface antigens Thy-1,
Lyt-2 and L3T4 (all from Becton Dickinson). Both Thy-1 and

Lyt-2 were fluorescein isothiocyanate (FITC) conjugated whereas L3T4 antibodies were phycoerythrin (PE) conjugated. With every flow cytometric analysis, we also labeled thymocytes with fluorescein diacetate (FDA, Becton Dickinson) to determine the population of red and dead cells remaining in the gated cell population. Before doing each analysis, the instrument was standardized by use of beads in a fluorescein/phycoerythrin compensation kit (Flow Cytometric Standard Corp., Research Triangle Park, NC).

Chronic Alcohol Exposure

Mice , Mating and Rearing

C57BL/6J (B6, H-2^b) mice were used in the chronic alcohol exposure experiment. C57BL/6J were purchased from Jackson Laboratory (Bar Harbor, ME). All the female B6 mice were age-matched, 8 weeks old, and were grouped 5-6 females in a cage. All mice were fed chow and water ad-libitum before diet feeding. The mouse room conditions were 12 hr light:12 hr dark cycle and the dark period was from 11:00 to 23:00 hr, temperature was maintained at 22 ± 1^0 C.

All the female mice were grouped with 2-3 B6 male mice (age 3-6 months) for mating. In order to have 55-60 mice closely matched for plug date, the mating period was continued for 5 to 6 days. Every day mice were checked for plugs at 10 am and 10 pm. Once a positive vaginal plug appeared, the

pregnant mouse was assigned randomly to 23% ethanol derived calories (EDC), pair-fed (PF) or foster groups (see diet section below). Both 23% EDC and PF group pregnant mice were fed chow and water until day 5 of pregnancy, then switched to their respective liquid diet (described below). The foster mother group was fed with chow and water ad-libitum throughout the experiment.

Within two days after birth 23% EDC and PF pups were switched to foster mothers for nursing for 4 weeks. Litter sizes were recorded for all litters. All the pups were also weighed at 4 weeks of age before weaning. Then pups of the same sex were assigned to different immune assay groups, using pups from as many litters as possible (>4) for each immune assay group and matching EDC and pair-fed groups as closely as possible for age. These mice were housed 2-3 together following weaning and fed with chow and water ad-libitum until sacrificed for immune assay.

Diet and Feeding

The mouse liquid diet was purchased from Bioserv Co. (Frenchtown, NJ). The diet regimen in the alcohol treatment group was 23% ethanol derived calories (EDC) which contained alcohol in a concentration such that 23% of the total calories were derived from ethanol. The pair-fed diet group was restricted in that their food and calorie consumption matched that of the 23% EDC group. The diet was an isocaloric control

diet in which maltose dextrin substituted for the calories contributed by ethanol in the 23% EDC diet. The foster group mice were fed with chow and water ad-libitum. The liquid diet used for feeding in the chronic alcohol exposure experiment was formulated to provide adequate nutrition for the pregnant mice and fetuses (89). The feeding procedure was modified by Ewald et al. (27) and also was used in our previous FAS studies. Thirty-five ml of fresh 23% ethanol derived calories diet were put in 50 ml conical tubes with stopper and sipper tube for feeding. PF control group mice were given isocaloric diet in the same amount as the mean amount eaten by 23% EDC group mice on the same day of pregnancy; therefore, PF groups were one day behind 23% EDC groups. All the feedings were from day 5 to day 19 of the gestation period. After recording the previous day diet consumption, fresh liquid diets were given in the morning between 9:00 am and 10:00 am. At gestational day 19, all mice on the liquid diets were switched to chow and water.

Blood Alcohol Levels (BAL)

To determine blood alcohol levels in the 23% EDC group animals, the method described above (section I) was used. Due to the teratogenic effects of metafane, a different group of nonpregnant age-matched mice fed with 23% EDC diet was used for the assays. Routinely, blood samples were drawn at 5 pm

(7 hr after receiving fresh food) on the days that correspond to gestational days 9, 13 and 18 for the 23% EDC group.

In one experiment, blood was drawn at the end of the light period before mice received fresh food and 2-pm (4 hr after fresh food) to see if blood alcohol levels differed at different times under this feeding procedure.

Spleen Cell Flow Cytometric Analysis

Spleen cells from both 23% EDC and PF groups mice of 6-8 weeks of age, were analyzed by flow cytometry for the expression of T cell and B cell differentiation antigens. Cells from the same preparations were also used to set up allogeneic mixed lymphocyte reactions (AMLR). Flow cytometric analysis was also used to assess the enrichment of various cell subpopulations in nylon wool adherent and non-adherent fractions which were subsequently used in syngeneic mixed lymphocyte reaction (SMLR).

The splenocyte preparation and labeling procedures were the same as the thymus cell procedure described above (Section I). PMEM (Section I) was used in flow cytometric assay. Hypotonic lysis was used to remove red blood cells from spleen lymphocyte preparations. MLC medium (see MLR section below) instead of PMEM was used to prepare the splenocyte suspensions, but in the final labeling cells were suspended in PMEM. To quantify B cells, anti-mouse IgG-FITC conjugated monoclonal antibody was used.

Plaque Forming Cell Assay (PFC)

DBSS, Dutton (61) balanced salt solution, pH 7.2 was used in the plaque forming cell assay (PFC). For thymus-dependent antigen, sheep red blood cell (SRBC; Colorado Serum Company, Denver, CO) and thymus independent antigen, bacterial lipopolysaccharide (LPS; RIBI Immunochem Research Inc., Hamilton, MT) extracted from Escherichia coli were used. In primary SRBC direct PFC assay (test which detects IgM secreting B cells) mice of each group were immunized by i.v. injection of 0.25 ml 10% suspension of sheep red blood cells (SRBC) 5 days before PFC assay.

In secondary SRBC PFC assay, mice were immunized with the same volume and concentration of SRBC as above by i.v. injection at 21 and 5 days before PFC assay. To calculate indirect (IgG) plaques, the PFC number from both direct and indirect assays were calculated first, then direct PFC number was subtracted from indirect PFC number to obtain IgG plaque number.

For LPS immunization schedule, mice were given a single i.v. injection of 0.25 ml (10 µg) of LPS 4 days before direct assay of LPS-specific PFC. When LPS-coated SRBC were used to estimate LPS-specific plaques, background anti-SRBC PFC numbers were subtracted from total PFC numbers.

The plaque-forming cell methods for primary and secondary SRBC and primary LPS as described by Ha et al. (39) were used.

Briefly, the slide modification of the localized hemolysis in gel technique used sheep red blood cells, or sheep red blood cells coated with LPS as indicator cells. A mixture of indicator cells and 5-fold serial dilutions of splenocytes in 0.65% sea-plaque agarose (FMC Bioproducts, Rockland, ME) were spread on 25 x 75 mm frosted microscopy slides (VWR Scientific Inc. San Francisco, CA). Guinea pig complement (Colorado Serum Company, Denver, CO) was absorbed with SRBC, then diluted (1:10) in DBSS for use in direct PFC assay. Complement diluted 1:10 (DBSS) and goat anti-mouse IgG diluted 1:50 in DBSS were used in the indirect PFC assay. After adding complement or complement plus anti-mouse IgG, slides were incubated at 37^0 C for 2 hr and then refrigerated over night. Plaques were counted after overnight incubation. The number of antigen-specific plaque-forming cells in spleens was expressed as total PFC/spleen and PFC/10^6 cells.

Delayed Type Hypersensitivity

Delayed-type hypersensitivity (DTH) assays were performed as described by Zidell et al. (93). Briefly, mice were sensitized to keyhole limpet hemocyanin, KLH, (Calbiochem, La Jolla, CA) by subcutaneous (s.c.) injection, into each inguinal region of 100 μg KLH emulsified in 0.1 ml of incomplete Freund's adjuvant (GIBCO, Detroit, MI). Six days after immunization, these mice received 0.2 ml of PBS containing 8 x 10^{-6} M 5-fluorodeoxyuridine (Sigma, St. Louis,

MO) i.p. Thirty min later, all the mice were injected with 0.2 ml of PBS containing 10 μCi/ml $[^{125}I]$-iododeoxyuridine ($[^{125}I]$ UdR, New England Nuclear, Boston, MA). Twenty-four hr later, ear thickness of right and left ears was measured using a dial thickness gauge engineer's micrometer (Mitutoyo Co. Tokyo, Japan). Then, all mice were challenged by intradermal injection of 20 μl of PBS containing 30 μg KLH (Calbiochem Co. La Jolla, CA) in the pinna of the right ear and PBS alone in the left ear. One day after challenge, each mouse was checked for ear thickness of right and left ears. The mice were sacrificed by cervical dislocation and the central portion of the ears was removed by 7 mm diameter punch. The plugs were weighed and put into scintillation tubes for radioactivity counting in a gamma counter (Packard, Laguna Hill, CA). The data were expressed as the ratio of challenged ear/non-challenged ear for ear weight, ear thickness and radioactivity count.

Contact Sensitivity

Contact sensitivity (CS) assays were performed by the method described by Mekori et al. (59). Briefly, mice were sensitized with 80 μl of 3% 4-ethoxymethylene-2-phenol oxazolone (OX; BDH Chemical, Poole, England) in 4:1 acetone:olive oil (v/v). Sixty μl aliquots were applied to the shaved area of the abdomen and 5 μl aliquots to each of the feet. After five days, all the mice were challenged with

10 µl of 0.5% OX solution on both sides of the right ear, whereas the left ear was challenged with vehicle solution only in the same pattern. Twenty-four hr later, the CS results were measured and expressed as described in DTH section above.

Mixed Lymphocyte Reaction

Allogeneic mixed lymphocyte reaction (AMLR)

AMLR assay was performed as described by Fitch et al. (85). Briefly, mice were killed by cervical dislocation and the spleens were removed aseptically in the MLC medium and were teased individually to single cell suspension. The mixed lymphocyte culture (MLC) medium used for allogeneic and syngeneic mixed lymphocyte reaction was RPMI 1640 (Irvine Scientific) containing 10% FCS (Hyclone) and 10mM HEPES, pH 7.2, N-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Irvine Scientific), 200 mM glutamine, 100 µg/ml penicillin, 75 µg/ml streptomycin, 2.5 µg/ml fungizone, 2gm/l sodium bicarbonate and 5×10^{-5} 2-mercaptoethanol.

The cell suspensions were treated by hypotonic lysis to remove red blood cells. The responder cells from C57BL/6J (H-2^b) mice were adjusted to 2.5×10^6 cells/ml in MLC-10% FCS. The BALB/c (H-2^d) spleen stimulator cells were adjusted to a concentration of 5×10^6 cells/ml after treating with 30µg/ml of mitomycin C (Sigma, St. Louis, MO) for 1 hr at 37^0 C in a 5% CO_2 incubator. Before adding to the AMLR, the stimulator

cells were washed four times over a cushion of FCS and resuspended in MLC with 10% FCS medium, then adjusted to 5 x 10^6 cells/ml. The assays were performed in triplicate for each sample in a total volume of 200 µl (5 x 10^5 cells/well of both effector and stimulator) for 3, 4, 5 and 6 days culture. Proliferation was determined by pulsing 1 µCi/well of [^{3}H]-thymidine (sp act 2 Ci/nM, New England Nuclear, Boston, MA) and incubating for 4 hr. Cells were harvested with a multiple automated PHD cell harvester (Cambridge Technology Inc., Watertown, MA). The data were expressed as actual counts per min (cpm).

Syngeneic mixed lymphocyte reaction (SMLR)

The SMLR assay was performed as described in Suzuki et al. (86) with some modification. Basically, cell suspensions were prepared from the pooled spleens of three mice (to get enough cells for nylon wool column separation) as described above (MLR section), then the cells were adjusted to a concentration of 2 x 10^7 cells/ml. The splenocyte suspensions were run through a nylon-wool column (62) to separate nylon-wool non-adherent cells (T cell enriched) which were used as responder cells and nylon-wool adherent cells (mostly B cell and macrophage enriched) which were used as stimulator cells in the SMLR. The responders were adjusted to 1 x 10^7 cells/ml and the stimulators were treated with mitomycin C (see MLR section), washed, and then adjusted to 5 x 10^6 cells/ml. SMLR

cultures were maintained, pulsed and counted as described as in the AMLR assay. Responder cells (1 x 10^6 cells/well) and stimulator cells (5 x 10^5 cells/well) were put in 96-well flat bottom microplates (Costar, Cambridge, MA).

Cytotoxicity Assays

Cytotoxic T cells were generated by allogeneic mixed lymphocyte reaction (AMLR, described as above) culture except cultures were in 2 ml volume containing 2.5 x 10^6 effector cells per well and 5 x 10^6 stimulator cells per well. After 4 days culture in a 24-well flat plate (Nunc, Roskilde, Denmark) at 37^0 C, 5% CO_2, cells were harvested, centrifuged and resuspended in a minimal volume to carry out a serial two-fold dilution of CTL .

Chromium release assay was performed as described in Harp et al. (41). Briefly, the cytotoxic T cells were put 100µl/well into the 96-well V-bottom microplates (Scientific Resource Associate, Bellevue, WA) with 100µl/well of ^{51}Cr-labeled P815 (H-2^d) target cells (1-2 x 10^4 cells/well). The P815 cell line, a mastocytoma of DBA/2 (H-2^d) origin, was used as the target in the chromium release assay. These cells were maintained in vitro by passage in RPMI 1640 (Cat. 9512, Irvine Scientific, Santa Ana, CA) medium supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM glutamine (Irvine Scientific) 5 x 10^{-5} M 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA), 100 µ/ml penicillin, 75 µg/ml streptomycin, 2.5 µg/ml

fungizone (Irvine Scientific). The labeling procedure for target cells was from Brunner et al. (14) with minor modifications. Target P815 cells were labeled with 100 µl $Na_2^{51}CrO_4$ for 90 min at 37^0 C, in a 5% CO_2 incubator, then the cells were washed four times by centrifugation over FCS and resuspended to a concentration of 1×10^5 cells/ml in MLC medium. Then, the assay plates were incubated at 37^0 C, 5% CO_2 for 4 hr before harvesting. The plates were centrifuged at 150 x g at room temperature for 3 min both before and at the end of incubation. One hundred microliters of the medium were removed from each well and placed in scintillation vials and counted in the gamma counter (Packard, Laguna Hill, CA). Values for each sample (triplicate) were expressed as percent specific cytotoxicity from the formula :

$$\frac{\text{Experimental } ^{51}\text{Cr release} - \text{Spontaneous } ^{51}\text{Cr release}}{\text{Maximum } ^{51}\text{Cr release} - \text{Spontaneous } ^{51}\text{Cr release}} \times 100\%$$

Spontaneous ^{51}Cr release was determined from the targets without effector cells and maximum ^{51}Cr release was from target cells treated with detergent, Zap-Oglobin (Coulter Diagnostic, Hialeah, FL). The lytic unit (LU) was defined as the cytotoxic activity required to achieve 50% specific lysis of the labeled target cells under the standard assay conditions. Both LU/culture and LU/10^6 viable cells were calculated after

viable cell recovery in 4 day AMLR culture had been determined. The number of LU/culture represented a relative measure of the number of CTL whereas the number of $LU/10^6$ cells represented a relative measure of the frequency of CTL in the various cell populations.

RCS Driven Proliferation of Con-A Blast T Cell Assay

RCS (rat Con-A supernatant) driven proliferation of Con-A blast T cell response was performed as described by Norman et al (66). Rat Con A supernatant (RCS, the unfractionated 42 hr supernatant of 2×10^7 rat spleen cells/ml cultured with 10 µg/ml Con-A) was used in the Con-A blast T cell proliferation assay. In brief, the cell suspension of each pooled spleen preparation was adjusted to a concentration less than 2×10^7 cells/ml before running separation over nylon-wool column, then enriched T cells (nylon-wool non-adherent cells) were adjusted to 2×10^6 cells/ml in 10 ml MLC-10% FCS with 2 µg/ml Con-A (Sigma, St. Louis, MO) for blastogenesis. After 60-64 hr of culture, the blast T cells were harvested and washed in Hank's balanced salt solution (HBSS) containing 10 mg/ml alpha-methylmannose to remove cell-bound Con-A. The washed cells were separated by centrifugation over a Lympholyte M gradient (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) at 700 x g, 20 min at room temperature to separate the blast T cells from other cells and dead cells. The blast T lymphocytes were collected from the interface and washed three

times with MLC-10% FCS before use in the Rat Con-A supernatant (RCS) driven proliferation experiment. RCS containing IL-2 was used at several dilutions with blast T cells in 96-well flat-bottom microplates (Costar, Cambridge, MA) and incubated 24 hr. All the assays were performed in triplicate and the proliferation was determined by 4 hr [^{3}H]-thymidine pulsing. The proliferation data were expressed as actual cpm count.

Statistical Analysis

Statistical comparisons between ethanol treated and control group values for each parameter were determined by an analysis of variance by using the statistical analysis package MSUSTAT 5.0 developed by R.E. Lund (Research and Development Institute, Inc., Montana State University, Bozeman, MT).

RESULTS

Acute Alcohol Exposure

Blood Alcohol Levels (BAL)

The mouse maternal blood alcohol level, after acute alcohol i.p. exposure, reflects the potential risk of fetuses to the circulating alcohol, because alcohol is a small molecule that freely passes through the placenta (22). Therefore, we monitored blood alcohol levels (in non-pregnant females) to determine the BAL following alcohol injection. In the acute alcohol experiment, the maternal blood alcohol levels were around 500 mg/100 ml after the first i.p. injection and above 600 mg/100 ml after the second i.p. injection. The same range of blood alcohol levels has also been reported by others using this method (91,92). The second blood alcohol level was higher than the first BAL, meaning that C57BL/6 mice could not metabolize all the alcohol from the first injection in 4 hr. In other words, the fetuses would have at least 8 hrs' alcohol exposure during the embryogenesis period. After each alcohol i.p. injection, the mice were in an ataxic state for several hr.

Effect of Maternal High Alcohol Exposure on Fetuses

Initially, we wished to confirm that the ethanol injection regimen of two doses of 2.9 g/kg alcohol exposure in

utero on one gestational day would induce the same pattern of FAS features in C57BL/6 mice as described by Webster at al (91,92). The results, in Table 1, indicated the average body weight of fetuses which were exposed to ethanol on gestational day 8, 9, 10, or 11 was significantly lower than the saline controls. After examination of fetuses for morphological abnormalities, we found 14 out of 29 fetuses (48%) in three litters with right fore-limb digits missing (4th and/or 5th digit) in the group that was exposed to ethanol on gestational day 9. A higher incidence of resorption (resulting in smaller litter size) was noted in gestational day 10 and gestational day 11 alcohol injected groups (50 %) than in saline control groups (0%). The litter size was normal for animals exposed to ethanol on gestational day 7, 8 and 9.

Effect of Acute Alcohol Exposure on Fetal Thymus

As described above, we found external physical features typical of fetal alcohol syndrome in the 18 day fetuses in the alcohol treatment group; the next question we intended to answer was whether acute alcohol exposure causes an abnormal thymus, such as small thymus or thymic aplasia. In the analysis of fetal thymuses, the results demonstrated that thymuses of 18-day fetuses in the alcohol treatment group were smaller than those of the saline control group for exposure on all gestational days. No missing thymus was noted in any of the fetuses from alcohol treated or saline treated groups.

Because in the ontogeny of the mouse thymus, function and immunocompetence are associated with thymocyte subpopulations with particular antigenic phenotypes, we analyzed fetal thymocytes by FACS for expression of L3T4, Lyt-2 and Thy-1 antigens. There was no significant difference between ethanol and saline exposure groups for thymocyte subpopulations, with the exception of day 10 exposure, in which too few pups were recovered in the alcohol exposure group to perform statistical analysis (Table 2).

Webster et al. (91) demonstrated that fetuses that were small were also the most likely to have other anomalies associated with FAS. Table 3 compares fetuses with weight less than 0.8 gm in ethanol treated groups with fetuses in the saline exposed groups. When only fetuses weighing <0.8 gm were considered, thymocyte number from the day 8 alcohol exposure group is considerably lower (46% of control) than when all fetuses from this exposure day are considered (79% of control). On the other hand, even fetuses weighing <0.8 gm in the day 9 exposure group had thymuses not much smaller (68% of control) than the average for all exposed fetuses from that day (76%). The majority of fetuses recovered from day 10 or day 11 alcohol exposure were very small and their thymocyte number was also markedly reduced.

Table 1. Status of fetuses of acute alcohol exposure experiment.

Gestation day[a] Injection	Pups & Litters	Litter Size (±SD)	Average Fetal Weight (gm) (±SD)	Fetal Malformation
7^E	$41^b/4^c$	7.8±1.3	0.92±0.05	N
8^E	47/5	9.4±2.1	$0.85±0.08^d$	N
8^S	29/3	9.7±1.2	0.94±0.06	N
9^E	47/5	9.4±0.6	$0.84±0.07^d$	Digits[e] Missing
9^S	20/2	10.0±1.4	0.92±0.05	N
10^E	4/4	1.0±1.2	$0.73±0.02^d$	Resorption[f]
10^S	10/1	10.0±0.0	0.94±0.06	N
11^E	12/2	6.0±2.8	$0.72±0.07^d$	Small Litter[g]
11^S	11/1	11.0±0.0	0.83±0.10	N

a. Gestational day plus 12 hr and 16 hr; E = Ethanol Group
                                         S = Saline Control
b. No. of fetuses
c. No. of litters
d. Differs from saline control at P <0.01
e. 4th and/or 5th digits missing on right fore-limb
f. Two out of four litters completely resorbed
g. Lower number of pups in litter

Table 2. Flow cytometric analysis of thymuses of 18 day fetuses acutely exposed to ethanol.

Gestation Day[a] Injection	Thymus Cell # ($* 10^6$) (±SD)	Thy-1† (%) (±SD)	L3T4† Lyt-2† (%) (±SD)	L3T4† Lyt-2$^-$ (%) (±SD)	L3T4$^-$ Lyt-2† (%) (±SD)	L3T4$^-$ Lyt-2$^-$ (%) (±SD)
7^E	3.48 (±1.56)	95.20 (±5.00)	65.16 (±5.31)	1.36 (±0.34)	10.28 (±2.47)	23.20 (±5.42)
8^E	2.97^b (±1.14)	96.81 (±1.59)	59.50 (±9.79)	1.27 (±0.26)	10.69 (±2.43)	28.56 (±7.44)
8^S	3.76 (±0.89)	97.07 (±0.90)	62.70 (±8.22)	1.63 (±0.46)	9.70 (±2.95)	25.97 (±6.28)
9^E	3.51^b (±0.77)	95.96 (±0.93)	64.60 (±4.74)	1.30 (±0.19)	8.88 (±1.22)	25.24 (±3.93)
9^S	4.56 (±1.17)	97.50 (±0.00)	72.70 (±0.57)	1.20 (±0.00)	7.85 (±1.20)	18.25 (±0.64)
10^E	1.85^b (±0.51)	97.10 (±0.14)	44.95 (±9.83)	1.30 (±0.28)	18.20 (±2.69)	35.60 (±7.35)
10^S	4.15 (±0.83)	98.20 (±0.00)	73.50 (±0.00)	1.20 (±0.00)	7.40 (±0.00)	17.90 (±0.00)
11^E	2.06^b (±0.64)	95.55 (±2.62)	56.05 (±12.52)	1.65 (±0.21)	14.80 (±6.08)	27.55 (±6.72)
11^S	3.36 (±1.03)	ND^c	ND	ND	ND	ND

a. Gestational day plus 12 hr and 16 hr
   E = two doses alcohol (2.9 g/kg body weight) by i.p. injection; S = two i.p. injections of saline
b. Differs from saline control at P <0.01 level
c. ND = not done

Table 3. Effects of acute alcohol exposure in utero in day-18
fetuses.

Gestational[a] Day (+ 12 hr & 16 hr)	No.[b] of Fetuses	Average Fetal Weight (±SD)	Average Fetal Thymocytes n x 10^6 (±SD)	Fetuses with Limb Defects	Fetuses with Athymia
7^E	$3[2]^X$	0.73 (±0.10)	1.68 (±0.87)	0	0
8^E	$14[5]^X$	0.72^c (±0.06)	1.72^c (±0.57)	0	0
8^S	29[3]	0.94 (±0.06)	3.76 (±0.89)	0	0
9^E	$14[4]^X$	0.75^c (±0.05)	3.10^c (±1.01)	14[3]	0
9^S	20[2]	0.92 (±0.05)	4.56 (±1.17)	0	0
10^E	$4[2]^X$	0.73^c (±0.02)	1.85^c (±0.51)	0	0
10^S	10[1]	0.94 (±0.06)	4.15 (±0.83)	0	0
11^E	$10[2]^X$	0.70^c (±0.05)	2.06^c (±0.64)	0	0
11^S	11[1]	0.83 (±0.10)	3.36 (±1.03)	0	0

a. Acute alcohol or saline exposure day,
   E : ethanol, x :fetal weight <0.8 g
   S : saline
b. n = number of fetuses [no. of litters]
c. Differs from saline control at P <0.01 level

Chronic Alcohol Exposure

Blood Alcohol Levels

In each feeding experiment, five non-pregnant females of the same age mice were used to do the blood alcohol level test. BAL were determined on blood obtained 6 hr after the start of the dark period, because mice are nocturnal, and tend to eat during the dark period. As seen in Table 4, most of the maternal blood alcohol levels on day 4 and 8 of feeding were around 200 mg/100 ml; on day 13, the BAL varied from 71.4 to 200.2 mg/100 ml. The BAL in this chronic alcohol exposure experiment was much lower than that seen in the acute alcohol exposure experiments. We also did BAL in the morning in mice that had been on the alcohol diet for 11 days to examine fluctuations during a different time of the 24 hr feeding schedule. The BAL in the morning (at the end of the light period) was 12 mg/100 ml.

Maternal and Pup Status

The average food consumption of 23% EDC and pair-fed mice was not significantly different in any of the three experiments (Table 5). Alcohol consumption was 20.4 g/kg/day, based on g.d. 19 when we obtained a maternal weight. With adequate nutrition (Table 5), the maternal weight gains from gestation day 0 to gestation day 19 were not significantly different between 23% EDC and PF pups (P > 0.05) except in

experiment 3 in which 23% EDC mice actually gained more than PF mice.

Table 4. Blood alcohol level in mice following chronic alcohol exposure to 23% EDC diet[a]

Exp No.	n[b]	Day 4 BAL mg/100ml (±SD)	Day 8 BAL mg/100ml (±SD)	Day 13 BAL mg/100ml (±SD)
1	5	282.2 (±6.2)	218.8 (±51.7)	71.4 (±53.3)
2	5	238.2 (±57.3)	185.2 (±52.6)	200.2 (±53.2)
3	5	198.9 (±103.1)	206.2 (±32.5)	82.8 (±51.0)

a. Bleedings were performed at 5 pm, 6 hr after the start of the dark period
b. n = number of mice

The average litter size was not different between groups. However, average 4 week old pup weights in the first and third feeding series were significantly different. We did not find any physical abnormalities in any of the pups from either alcohol treated or saline control groups. However, pups in each group that were unusually small did not survive to 4 weeks of age. There was no consistent difference between EDC and PF groups in pup mortality (Table 6). The rather high

mortality of both groups may be in part be due to failure of
foster mothers to accept pups.

Table 5. Maternal and pup status of chronic 23% EDC liquid
diet and pair-fed diet feeding experiments.

Experiment No.	Diet Group	Maternal Diet Consumption (ml/day)[a] (±SD)	Maternal Weight Gain (gm)[b] (±SD)	Litter Size (±SD)	Average Pup Weight (gm)[c] M. (±SD)	F (±SD)
1	23% EDC[d]	17.0 (±3.7)	17.8 (±1.3)	6.0 (±1.5)	13.4[e] (±3.1)	11.3[f] (±2.2)
1	PF[g]	17.8 (±2.8)	18.2 (±2.0)	7.0 (±1.4)	16.1 (±2.3)	13.5 (±1.9)
2	23% EDC	17.6 (±4.3)	14.3 (±2.6)	5.8 (±2.4)	15.8 (±1.7)	13.4 (±0.9)
2	PF	17.6 (±3.7)	13.6 (±1.8)	7.5 (±1.4)	14.6 (±2.7)	13.0 (±1.3)
3	23% EDC	17.4 (±4.6)	16.2[e] (±2.0)	6.5 (±1.3)	14.4[f] (±2.0)	12.5[f] (±1.8)
3	PF	18.3 (±4.3)	13.9 (±3.1)	5.5 (±1.7)	16.0 (±1.4)	4.2 (±0.8)

a. Measured over gestational period day 5 to day 19
b. Pregnant female mice were weighed on gestation day 0 and
   day 19.
c. Pups were weighed at 4 weeks old before weaning
   M: male, F: female
d. 23% EDC - 23% ethanol derived calories
e. Differs from PF at P <0.05 level
f. Differs from PF at P <0.01 level
g. PF - Pair-fed against 23% EDC

Table 6. Pup survival data in three series of ethanol and
pair-fed liquid diet feeding experiments.

Experiment No.	Diet Group	Total No. at Birth (Litter)		Total No. Deaths	Mortality Rate
1	23% EDC[a]	36	(6)[c]	17	47.2%
1	PF[b]	56	(7)	7	12.5%
2	23% EDC	99	(17)	40	40.4%
2	PF	59	(8)	23	39.0%
3	23% EDC	78	(12)	18	23.1%
3	PF	55	(10)	13	23.6%

a. 23% EDC - 23% ethanol derived alcohol
b. PF - Pair-fed against 23% EDC
c. Number of litters of each experiment

Immunological Assays

Spleen Lymphocyte Populations in Young Adult Mice

We were interested in whether prenatal alcohol exposure
led to altered ratios of T and B subpopulations in young adult
mice. To determine this, we analyzed spleen cell populations
from mice aged 6-8 weeks for expression of L3T4, Lyt-2 and
Thy-1 antigens. We also used surface IgG (a B lymphocyte
marker) to detect the number of B cells. No significant
differences were noted between alcohol-treated and control

groups in IgG†, Thy-1^1, L3T4† or Lyt-2† cell populations (Table 7).

Table 7. Flow cytometric analysis of spleen cells from adult mice exposed to alcohol in utero and pair-fed controls.

Exp. No.	Exp. Treatment	Ig-G† (%) ($\pm$SD)	L3T4† (%) ($\pm$SD)	Lyt-2† (%) ($\pm$SD)	Thy-1† (%) ($\pm$SD)
1	23% EDC[a]	66.2 ($\pm$1.7)	17.8 ($\pm$1.1)	10.7 ($\pm$0.4)	31.2 ($\pm$2.2)
1	PF[b]	64.8 ($\pm$0.6)	17.9 ($\pm$1.4)	10.2 ($\pm$0.5)	33.4 ($\pm$1.5)
2	23% EDC	52.5 ($\pm$7.3)	20.8 ($\pm$1.4)	12.2 ($\pm$0.9)	36.7 ($\pm$2.8)
2	PF	52.9 ($\pm$2.8)	20.7 ($\pm$1.8)	11.5 ($\pm$0.9)	36.3 ($\pm$4.4)

a. 23% EDC, 23% ethanol derived calories
b. PF, pair-fed against 23% EDC

Plaque Forming Cell Assay

The in vivo hemolytic PFC assay allowed us to examine the humoral immune response to see whether the young adult mice with prenatal alcohol exposure still had normal antibody production ability. Six mice in each group were immunized with SRBC (a thymus dependent antigen), and LPS (extracted from E. coli, a thymus independent antigen) at the age of 6-8 weeks and tested for PFC. In addition, we tested the primary immune

response (IgM) and the secondary immune response (IgG) to SRBC. As seen in Table 8, the results showed that the total spleen cell number of offspring from the 23% EDC group was significantly higher than the PF group in two of five experiments. There was not a significant difference in the in vivo PFC response to either thymus dependent or thymus independent antigens based on total PFC / spleen or PFC / 10^6 cells.

DTH and CS Immune Assay

A very recent report by Gottesfeld et al.(37) demonstrated that fetal alcohol exposure could induce depression in the contact sensitivity response to TNCB (trinitrochlorobenzene) of adult mice. On the contrary, Zidell et al. (93) claimed that there was no change in the delayed type hypersensitivity reaction to KLH in similarly exposed mice. Therefore, we decided to investigate both these T cell mediated immune responses in 8 week old mice which had been exposed to alcohol in utero. The results (Table 9) showed, with one exception, no significant difference between 23% EDC and PF groups in DTH and CS in vivo immune response in the measurement of ear thickness ratio, ear weight ratio and ear ^{125}I-UdR ratio. The ear thickness ratio was significantly different in the second DTH experiment, in which the value was significantly higher for the PF than for the 23% EDC group. However, other measures of DTH (ear weight ratio and ear

$[^{125}I]$-UdR ratio) in the same animals were not significantly different.

Table 8. Antigen specific IgG and IgM plaque forming cell assays of mice from chronic alcohol exposure and control groups[a].

Treatment	Antigen /Class	Sex[b]	Spleen Cell # (x 10^6) (±SD)	PFC # (x 10^3) (±SD)	PFC/10^6 Cells (±SD)
23% EDC[c]	SRBC[d]/IgM	F	165.4[e] (±18.8)	151.1 (±38.2)	914.3 (±229.0)
PF[f]	SRBC/IgM	F	122.2 (±37.2)	159.0 (±71.4)	1304.3 (±609.8)
23% EDC	SRBC/IgM	F	161.0 (±33.4)	182.7 (±60.5)	1121.0 (±225.6)
PF	SRBC/IgM	F	142.7 (±20.3)	195.0 (±38.6)	1392.0 (±339.6)
23% EDC	LPS[g]/IgM	M	155.2 (±26.7)	4.2 (±1.6)	26.5 (±9.4)
PF	LPS/IgM	M	126.0 (±23.3)	4.2 (±1.8)	33.7 (±15.7)
23% EDC	LPS/IgM	M	156.5 (±25.9)	8.3 (±3.2)	52.0 (±13.9)
PF	LPS/IgM	M	161.8 (±18.5)	7.6 (±4.5)	46.4 (±25.4)
23% EDC	SRBC/IgG	F	184.5[e] (±13.9)	138.0 (±30.4)	747.7 (±147.9)
PF	SRBC/IgG	F	155.8 (±25.1)	112.8 (±23.5)	774.8 (±205.2)

a. n = 6 animals for each group, except in first experiment where n = 5.
b. F,female, M,male.
c. 23% EDC, 23% ethanol derived calories
d. SRBC, sheep red blood cells
e. Differs from PF at P <0.05 level
f. PF, pair-fed against 23% EDC mice
g. LPS, lipopolysaccharide extracted from E. coli.

Table 9. DTH response and CS response of 6-8 week old
offspring from chronic alcohol exposure and control
groups[a].

Exp. No.	Diet Group	Immune Response	Sex[b]	Ear Thickness Ratio (R/L)[c] (±SE)	Ear Weight Ratio (R/L)[c] (±SE)	Ear [^{125}I]-UdR Ratio (R/L)[c] (±SE)
1	23% EDC[d]	DTH/KLH[e]	F	1.85 (±0.22)	2.10 (±0.41)	5.03 (±0.78)
1	PF[f]	DTH/KLH	F	1.73 (±0.47)	1.94 (±0.45)	4.23 (±2.09)
2	23% EDC	DTH/KLH	F	1.60[g] (±0.30)	1.85 (±0.57)	5.97 (±3.07)
2	PF	DTH/KLH	F	2.10 (±0.25)	2.37 (±0.29)	7.35 (±1.96)
3	23% EDC	CS/Oxa[h]	M	2.07 (±0.39)	1.98 (±0.35)	4.50 (±1.62)
3	PF	CS/Oxa	M	2.10 (±0.57)	1.98 (±0.54)	3.64 (±1.57)

a. n = 6 mice/group, except exp.1, 23% EDC, where n = 5
b. Sex, F-female, M-male
c. R/L, R-right ear,L-left ear
d. 23% EDC, 23% ethanol derived calories
e. KLH,keyhole limpet hemocyanin
f. PF, pair-fed against 23% EDC
g. Differs from PF at P <0.05 level
h. Oxa, oxazolone

Mixed Lymphocyte Reaction

After we did PFC, DTH and CS in vivo immune assays, we
performed in vitro immune tests to compare cellular immune
response of splenocytes from mice in the two groups. The
allogeneic mixed lymphocyte reaction (AMLR) assay provided a
test for T lymphocyte proliferation of experimental (B6, H-2[b])
spleen cells after mixing with allogeneic BALB/c (H-2[d]) mouse

spleen cells.   In this assay, spleen cells from 6 to 8 week old mice from EDC and PF groups were tested for proliferation ability after 3,4,5 and 6 days of mixed lymphocyte reaction. As shown in Figure 1, spleen cells from the 23% EDC group had significantly higher ^{3}H-thymidine incorporation than those from the PF group.   On the other hand, in the second and third AMLR experiments (Figures 2 and 3), there was no significant difference between 23% EDC and PF groups.

In syngeneic mixed lymphocyte reaction (SMLR) assay, we used pooled spleens for nylon wool column separation to get enough cells for this experiment. Figure 4 and Figure 5 showed no consistent pattern in the relative response of the two diet groups.   In the first experiment, the 23% EDC group had a better immune response than the PF group, yet, in the second experiment, the PF group had a higher ^{3}H-thymidine incorporation than the 23% EDC group.