



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  
© Copyright by Ching Huang (1990)

**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.

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  
Bozeman, Montana

December 1990

© COPYRIGHT

by

Ching Huang

1990

All Rights Reserved

N378  
H8598

APPROVAL

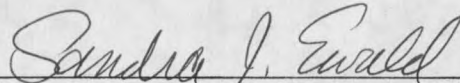
of a thesis submitted by

Ching Huang

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

10-30-90

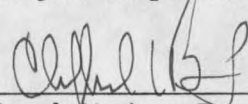
Date

  
Chairperson, Graduate Committee

Approved for the Major Department

10-26-90

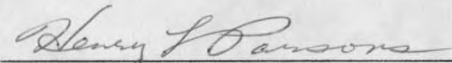
Date

  
Head, Major Department

Approved for the College of Graduate Studies

11/28/90

Date

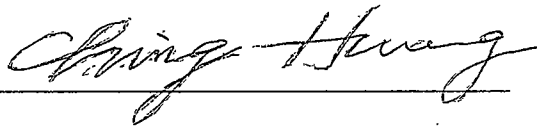
  
Graduate Dean

## STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made.

Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted by the copyright holder.

Signature



Date

Oct 25 1990

## ACKNOWLEDGMENTS

I would especially like to acknowledge Dr. Sandra J. Ewald for the exceptional guidance and inspiration that was rendered during the course of this thesis. I would also like to acknowledge Dr. Norman D. Reed and Dr. Cliff Bond for their helpful discussions and guidance. Lastly I wish to thank Connie McDonald for technical help in my study and my parents for their interest and support.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS.....	iv
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABSTRACT.....	ix
INTRODUCTION.....	1
MATERIALS AND METHODS.....	12
Acute Alcohol Exposure.....	12
Mice and Mating.....	12
Acute Alcohol Administration.....	12
Physical Examination of Fetuses.....	13
Blood Alcohol Levels.....	13
Flow Cytometric Analysis of Thymus Cells.....	14
Chronic Alcohol Exposure.....	15
Mice, Mating and Rearing.....	15
Diet and Feeding.....	16
Blood Alcohol Levels.....	17
Spleen Cell Flow Cytometric Analysis.....	18
Plaque-Forming Cell Assay.....	19
Delayed-Type Hypersensitivity.....	20
Contact Sensitivity.....	21
Mixed Lymphocyte Reaction.....	22
Cytotoxicity Assays.....	24
RCS Driven Proliferation of Con-A Blast T Cell Assay.....	26
Statistical Analysis.....	27
RESULTS.....	28
Acute Alcohol Exposure.....	28
Blood Alcohol Levels.....	28
Effect of Maternal High Alcohol Exposure On Fetuses.....	28
Effect of Acute Alcohol Exposure on Fetal Thymus.....	29
Chronic Alcohol Exposure.....	34
Blood Alcohol Levels.....	34
Maternal and Pup Status.....	34

## TABLE OF CONTENTS - Continued

Immunological Assays.....	37
Spleen Lymphocyte Populations in Young Adult Mice.....	37
Plaque Forming Cell Assay.....	38
DTH and CS Immune Assay.....	39
Mixed Lymphocyte Reaction.....	41
Cytotoxic T Lymphocyte Responses.....	48
RCS Driven Proliferation of Con-A Blasts.....	48
DISCUSSION.....	52
REFERENCES CITED.....	61



## LIST OF TABLES

Table	Page
1. Status of fetuses of acute alcohol exposure experiment.....	31
2. Flow cytometric analysis of thymuses of 18 day fetuses acutely exposed to ethanol.....	32
3. Effects of acute alcohol exposure in utero in day-18 fetuses.....	33
4. Blood alcohol level in mice following chronic alcohol exposure to 23% EDC diet.....	35
5. Maternal and pup status of chronic 23% EDC liquid diet and pair-fed diet feeding experiments.....	36
6. Pup survival data in three series of ethanol and pair-fed liquid diet feeding experiments.....	37
7. Flow cytometric analysis of spleen cells from adult mice exposed to alcohol in utero and pair-fed controls.....	38
8. Antigen specific IgG and IgM plaque forming cell assays of mice from chronic alcohol exposure and control groups.....	40
9. DTH response and CS response of 6-8 week offspring from chronic alcohol exposure and control groups.....	41
10. Generation of CTL activity in 4 day allogeneic mixed lymphocyte culture.....	49

## LIST OF FIGURES

Figure	Page
1. Allogeneic mixed lymphocyte reaction 1.....	43
2. Allogeneic mixed lymphocyte reaction 2.....	44
3. Allogeneic mixed lymphocyte reaction 3.....	45
4. Syngeneic mixed lymphocyte reaction 1.....	46
5. Syngeneic mixed lymphocyte reaction 2.....	47
6. RCS driven proliferation of Con-A blasts 1.....	50
7. RCS driven proliferation of Con-A blasts 2.....	51

## 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, cardiomasculature 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, Lyl 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<sup>b</sup>) 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  $22 \pm 1^{\circ}$  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  $\text{Na}_2\text{HPO}_4$ , 0.001M  $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$  and adjusted to pH 7.0. This medium was sterilized by filtration through a 0.22  $\mu\text{m}$  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  $2 \times 10^7$  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<sup>b</sup>) 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  $22 \pm 1^{\circ}$  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° 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<sup>6</sup> 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<sup>-6</sup> 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  $\mu\text{Ci/ml}$  [ $^{125}\text{I}$ ]-iododeoxyuridine ([ $^{125}\text{I}$ ] 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  $\mu\text{l}$  of PBS containing 30  $\mu\text{g}$  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  $\mu\text{l}$  of 3% 4-ethoxymethylene-2-phenol oxazolone (OX; BDH Chemical, Poole, England) in 4:1 acetone:olive oil (v/v). Sixty  $\mu\text{l}$  aliquots were applied to the shaved area of the abdomen and 5  $\mu\text{l}$  aliquots to each of the feet. After five days, all the mice were challenged with



10  $\mu$ l 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  $\mu$ g/ml penicillin, 75  $\mu$ g/ml streptomycin, 2.5  $\mu$ g/ml fungizone, 2gm/l sodium bicarbonate and  $5 \times 10^{-5}$  2-mercaptoethanol.

The cell suspensions were treated by hypotonic lysis to remove red blood cells. The responder cells from C57BL/6J (H-2<sup>b</sup>) mice were adjusted to  $2.5 \times 10^6$  cells/ml in MLC-10% FCS. The BALB/c (H-2<sup>d</sup>) spleen stimulator cells were adjusted to a concentration of  $5 \times 10^6$  cells/ml after treating with 30 $\mu$ g/ml of mitomycin C (Sigma, St. Louis, MO) for 1 hr at 37<sup>o</sup> C in a 5% CO<sub>2</sub> 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  $5 \times 10^6$  cells/ml. The assays were performed in triplicate for each sample in a total volume of 200  $\mu$ l ( $5 \times 10^5$  cells/well of both effector and stimulator) for 3, 4, 5 and 6 days culture. Proliferation was determined by pulsing 1  $\mu$ Ci/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  $2 \times 10^7$  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  $1 \times 10^7$  cells/ml and the stimulators were treated with mitomycin C (see MLR section), washed, and then adjusted to  $5 \times 10^6$  cells/ml. SMLR

cultures were maintained, pulsed and counted as described as in the AMLR assay. Responder cells ( $1 \times 10^6$  cells/well) and stimulator cells ( $5 \times 10^5$  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  $2.5 \times 10^6$  effector cells per well and  $5 \times 10^6$  stimulator cells per well. After 4 days culture in a 24-well flat plate (Nunc, Roskilde, Denmark) at  $37^\circ$  C, 5%  $\text{CO}_2$ , 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 100 $\mu$ l/well into the 96-well V-bottom microplates (Scientific Resource Associate, Bellevue, WA) with 100 $\mu$ l/well of  $^{51}\text{Cr}$ -labeled P815 (H-2<sup>d</sup>) target cells ( $1-2 \times 10^4$  cells/well). The P815 cell line, a mastocytoma of DBA/2 (H-2<sup>d</sup>) 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)  $5 \times 10^{-5}$  M 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA), 100  $\mu$ /ml penicillin, 75  $\mu$ g/ml streptomycin, 2.5  $\mu$ g/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  $\mu$ l  $\text{Na}_2^{51}\text{CrO}_4$  for 90 min at 37 $^\circ$  C, in a 5%  $\text{CO}_2$  incubator, then the cells were washed four times by centrifugation over FCS and resuspended to a concentration of  $1 \times 10^5$  cells/ml in MLC medium. Then, the assay plates were incubated at 37 $^\circ$  C, 5%  $\text{CO}_2$  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  $^{51}\text{Cr}$  release was determined from the targets without effector cells and maximum  $^{51}\text{Cr}$  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  $2 \times 10^7$  rat spleen cells/ml cultured with 10  $\mu$ g/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  $2 \times 10^7$  cells/ml before running separation over nylon-wool column, then enriched T cells (nylon-wool non-adherent cells) were adjusted to  $2 \times 10^6$  cells/ml in 10 ml MLC-10% FCS with 2  $\mu$ g/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 [<sup>3</sup>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 et 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 <sup>a</sup> Injection	Pups & Litters	Litter Size ( $\pm$ SD)	Average Fetal Weight (gm) ( $\pm$ SD)	Fetal Malformation
7 <sup>E</sup>	41 <sup>b</sup> /4 <sup>c</sup>	7.8 $\pm$ 1.3	0.92 $\pm$ 0.05	N
8 <sup>E</sup>	47/5	9.4 $\pm$ 2.1	0.85 $\pm$ 0.08 <sup>d</sup>	N
8 <sup>S</sup>	29/3	9.7 $\pm$ 1.2	0.94 $\pm$ 0.06	N
9 <sup>E</sup>	47/5	9.4 $\pm$ 0.6	0.84 $\pm$ 0.07 <sup>d</sup>	Digits <sup>e</sup> Missing
9 <sup>S</sup>	20/2	10.0 $\pm$ 1.4	0.92 $\pm$ 0.05	N
10 <sup>E</sup>	4/4	1.0 $\pm$ 1.2	0.73 $\pm$ 0.02 <sup>d</sup>	Resorption <sup>f</sup>
10 <sup>S</sup>	10/1	10.0 $\pm$ 0.0	0.94 $\pm$ 0.06	N
11 <sup>E</sup>	12/2	6.0 $\pm$ 2.8	0.72 $\pm$ 0.07 <sup>d</sup>	Small Litter <sup>g</sup>
11 <sup>S</sup>	11/1	11.0 $\pm$ 0.0	0.83 $\pm$ 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 <sup>a</sup> Injection	Thymus Cell # (* 10 <sup>6</sup> ) (±SD)	Thy-1 <sup>+</sup> ( % ) (±SD)	L3T4 <sup>+</sup> Lyt-2 <sup>+</sup> ( % ) (±SD)	L3T4 <sup>+</sup> Lyt-2 <sup>-</sup> ( % ) (±SD)	L3T4 <sup>-</sup> Lyt-2 <sup>+</sup> ( % ) (±SD)	L3T4 <sup>-</sup> Lyt-2 <sup>-</sup> ( % ) (±SD)
7 <sup>E</sup>	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 <sup>E</sup>	2.97 <sup>b</sup> (±1.14)	96.81 (±1.59)	59.50 (±9.79)	1.27 (±0.26)	10.69 (±2.43)	28.56 (±7.44)
8 <sup>S</sup>	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 <sup>E</sup>	3.51 <sup>b</sup> (±0.77)	95.96 (±0.93)	64.60 (±4.74)	1.30 (±0.19)	8.88 (±1.22)	25.24 (±3.93)
9 <sup>S</sup>	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 <sup>E</sup>	1.85 <sup>b</sup> (±0.51)	97.10 (±0.14)	44.95 (±9.83)	1.30 (±0.28)	18.20 (±2.69)	35.60 (±7.35)
10 <sup>S</sup>	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 <sup>E</sup>	2.06 <sup>b</sup> (±0.64)	95.55 (±2.62)	56.05 (±12.52)	1.65 (±0.21)	14.80 (±6.08)	27.55 (±6.72)
11 <sup>S</sup>	3.36 (±1.03)	ND <sup>c</sup>	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 <sup>a</sup> Day (+ 12 hr & 16 hr)	No. <sup>b</sup> of Fetuses	Average Fetal Weight (±SD)	Average Fetal Thymocytes n x 10 <sup>6</sup> (±SD)	Fetuses with Limb Defects	Fetuses with Athymia
7 <sup>E</sup>	3[2] <sup>x</sup>	0.73 (±0.10)	1.68 (±0.87)	0	0
8 <sup>E</sup>	14[5] <sup>x</sup>	0.72 <sup>c</sup> (±0.06)	1.72 <sup>c</sup> (±0.57)	0	0
8 <sup>S</sup>	29[3]	0.94 (±0.06)	3.76 (±0.89)	0	0
9 <sup>E</sup>	14[4] <sup>x</sup>	0.75 <sup>c</sup> (±0.05)	3.10 <sup>c</sup> (±1.01)	14[3]	0
9 <sup>S</sup>	20[2]	0.92 (±0.05)	4.56 (±1.17)	0	0
10 <sup>E</sup>	4[2] <sup>x</sup>	0.73 <sup>c</sup> (±0.02)	1.85 <sup>c</sup> (±0.51)	0	0
10 <sup>S</sup>	10[1]	0.94 (±0.06)	4.15 (±0.83)	0	0
11 <sup>E</sup>	10[2] <sup>x</sup>	0.70 <sup>c</sup> (±0.05)	2.06 <sup>c</sup> (±0.64)	0	0
11 <sup>S</sup>	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<sup>a</sup>

Exp No.	n <sup>b</sup>	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) <sup>a</sup>	Maternal Weight Gain (gm) <sup>b</sup>	Litter Size	Average Pup Weight (gm) <sup>c</sup>	
					M.	F
		(±SD)	(±SD)	(±SD)	(±SD)	(±SD)
1	23% EDC <sup>d</sup>	17.0 (±3.7)	17.8 (±1.3)	6.0 (±1.5)	13.4 <sup>e</sup> (±3.1)	11.3 <sup>f</sup> (±2.2)
1	PF <sup>g</sup>	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 <sup>e</sup> (±2.0)	6.5 (±1.3)	14.4 <sup>f</sup> (±2.0)	12.5 <sup>f</sup> (±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 <sup>a</sup>	36 (6) <sup>c</sup>	17	47.2%
1	PF <sup>b</sup>	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<sup>+</sup>, Thy-1<sup>+</sup>, L3T4<sup>+</sup> or Lyt-2<sup>+</sup> 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 <sup>+</sup> (%) (±SD)	L3T4 <sup>+</sup> (%) (±SD)	Lyt-2 <sup>+</sup> (%) (±SD)	Thy-1 <sup>+</sup> (%) (±SD)
1	23% EDC <sup>a</sup>	66.2 (±1.7)	17.8 (±1.1)	10.7 (±0.4)	31.2 (±2.2)
1	PF <sup>b</sup>	64.8 (±0.6)	17.9 (±1.4)	10.2 (±0.5)	33.4 (±1.5)
2	23% EDC	52.5 (±7.3)	20.8 (±1.4)	12.2 (±0.9)	36.7 (±2.8)
2	PF	52.9 (±2.8)	20.7 (±1.8)	11.5 (±0.9)	36.3 (±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  $^{125}\text{I}$ -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

[<sup>125</sup>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<sup>a</sup>.

Treatment	Antigen /Class	Sex <sup>b</sup>	Spleen Cell # (x 10 <sup>6</sup> ) (±SD)	PFC # (x 10 <sup>5</sup> ) (±SD)	PFC/10 <sup>6</sup> Cells (±SD)
23% EDC <sup>c</sup>	SRBC <sup>d</sup> /IgM	F	165.4 <sup>e</sup> (±18.8)	151.1 (±38.2)	914.3 (±229.0)
PF <sup>f</sup>	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 <sup>g</sup> /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 <sup>e</sup> (±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<sup>a</sup>.

Exp. No.	Diet Group	Immune Response	Sex <sup>b</sup>	Ear Thickness Ratio (R/L) <sup>c</sup> ( $\pm$ SE)	Ear Weight Ratio (R/L) <sup>c</sup> ( $\pm$ SE)	[ <sup>125</sup> I]-UdR Ratio (R/L) <sup>c</sup> ( $\pm$ SE)
1	23% EDC <sup>d</sup>	DTH/KLH <sup>e</sup>	F	1.85 ( $\pm$ 0.22)	2.10 ( $\pm$ 0.41)	5.03 ( $\pm$ 0.78)
1	PF <sup>f</sup>	DTH/KLH	F	1.73 ( $\pm$ 0.47)	1.94 ( $\pm$ 0.45)	4.23 ( $\pm$ 2.09)
2	23% EDC	DTH/KLH	F	1.60 <sup>g</sup> ( $\pm$ 0.30)	1.85 ( $\pm$ 0.57)	5.97 ( $\pm$ 3.07)
2	PF	DTH/KLH	F	2.10 ( $\pm$ 0.25)	2.37 ( $\pm$ 0.29)	7.35 ( $\pm$ 1.96)
3	23% EDC	CS/Oxa <sup>h</sup>	M	2.07 ( $\pm$ 0.39)	1.98 ( $\pm$ 0.35)	4.50 ( $\pm$ 1.62)
3	PF	CS/Oxa	M	2.10 ( $\pm$ 0.57)	1.98 ( $\pm$ 0.54)	3.64 ( $\pm$ 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<sup>b</sup>) spleen cells after mixing with allogeneic BALB/c (H-2<sup>d</sup>) 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  $^3\text{H}$ -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  $^3\text{H}$ -thymidine incorporation than the 23% EDC group.

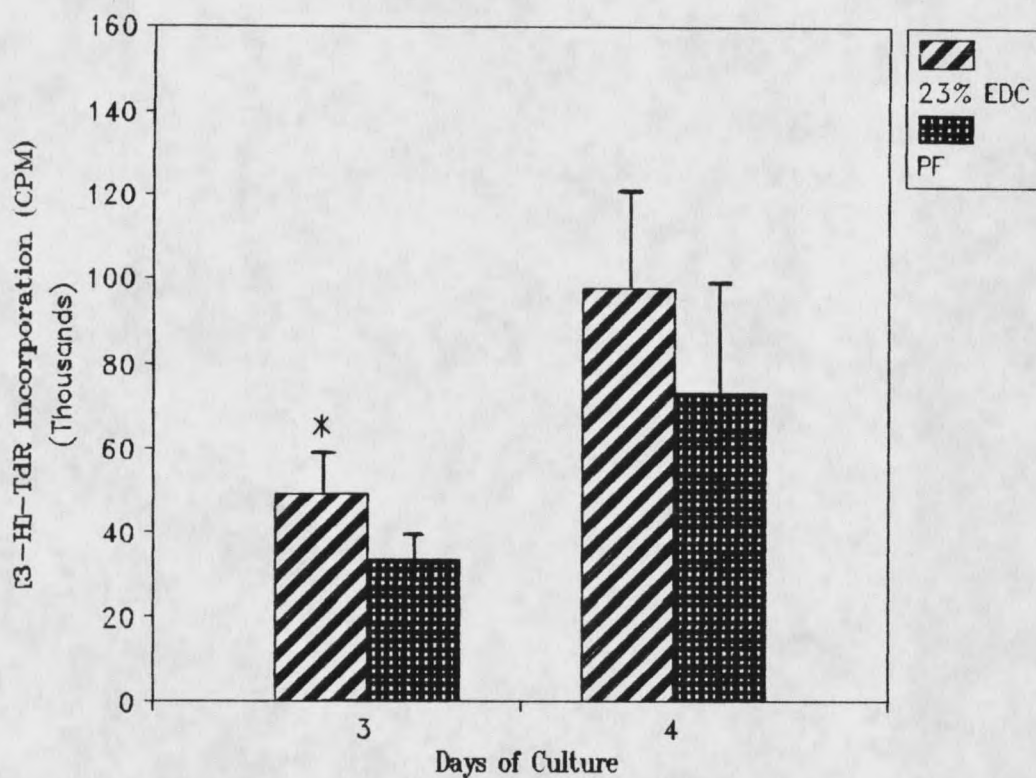


Figure 1. Allogeneic mixed lymphocyte reaction measured after 3 and 4 days culture. Proliferation response of effector cells, C57BL/6J splenocytes ( $5 \times 10^5$  cells/well) and BALB/c stimulator cells ( $5 \times 10^5$  cells/well) treated with mitomycin C.  
\* Differs from PF at P < 0.05 level

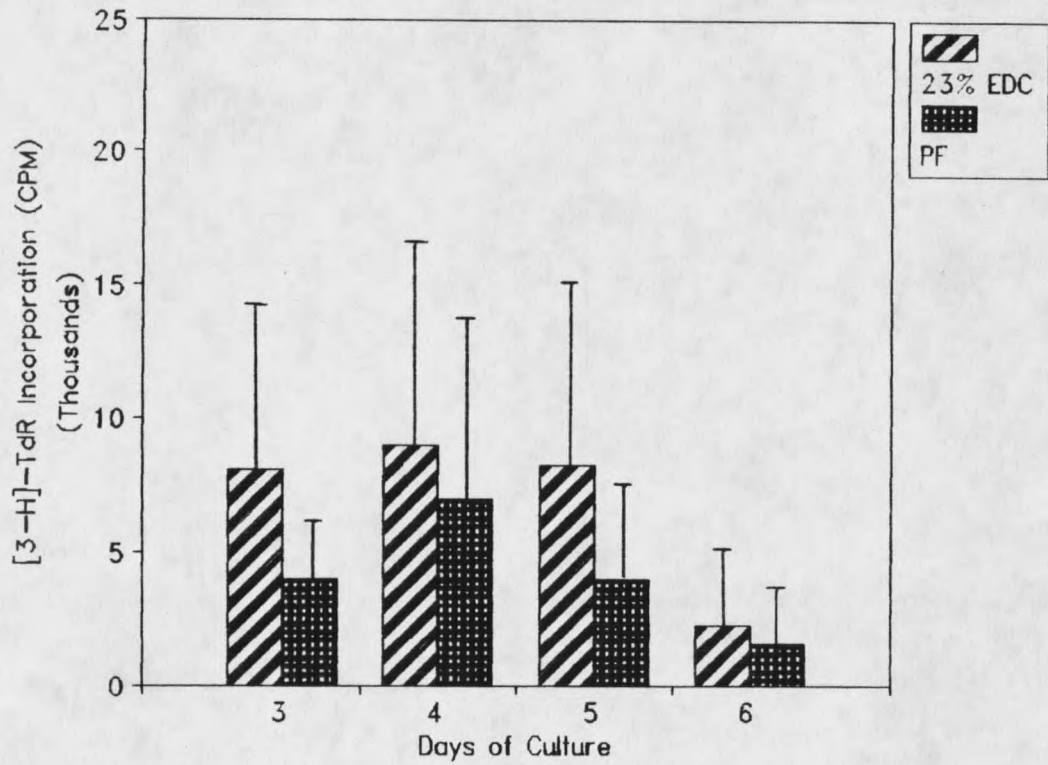


Figure 2. Allogeneic mixed lymphocyte reaction measured after 3, 4, 5 and 6 days culture. Proliferation response of effector cells, C57BL/6J splenocytes ( $5 \times 10^5$  cells/well) and BALB/c stimulator cells ( $5 \times 10^5$  cells/well) treated with mitomycin C.

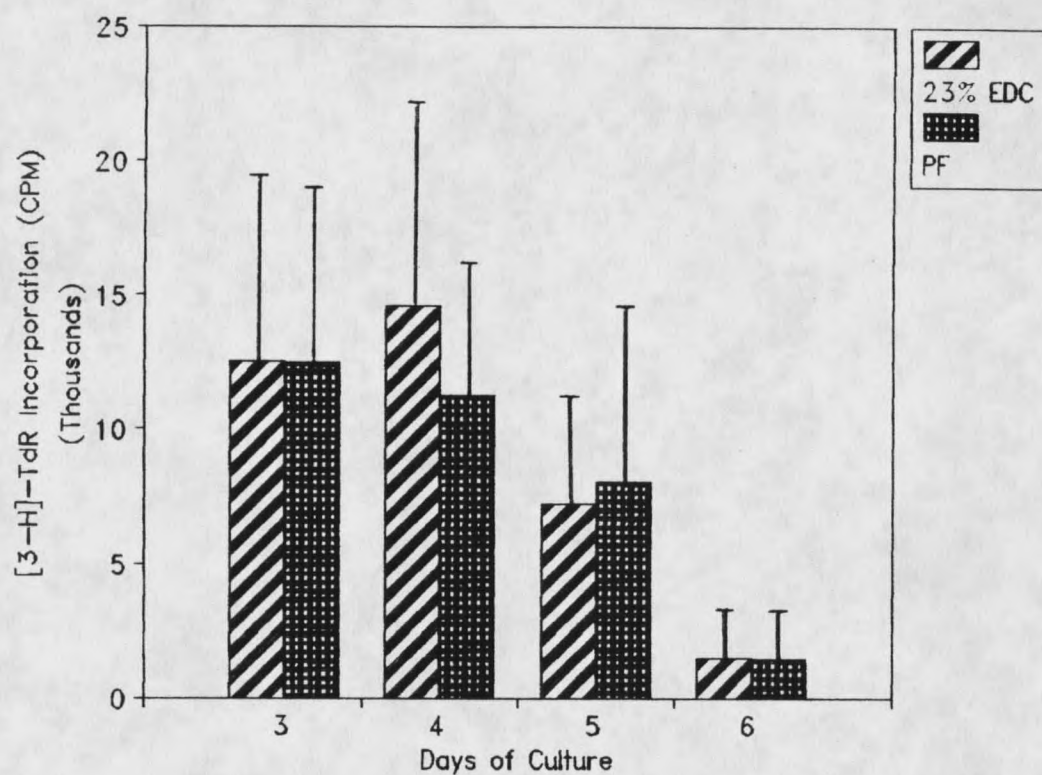


Figure 3. Allogeneic mixed lymphocyte reaction measured after 3, 4, 5 and 6 days culture. Proliferation response of effector cells, C57BL/6J splenocytes ( $5 \times 10^5$  cells/well) and BALB/c stimulator cells ( $5 \times 10^5$  cells/well) treated with mitomycin C.



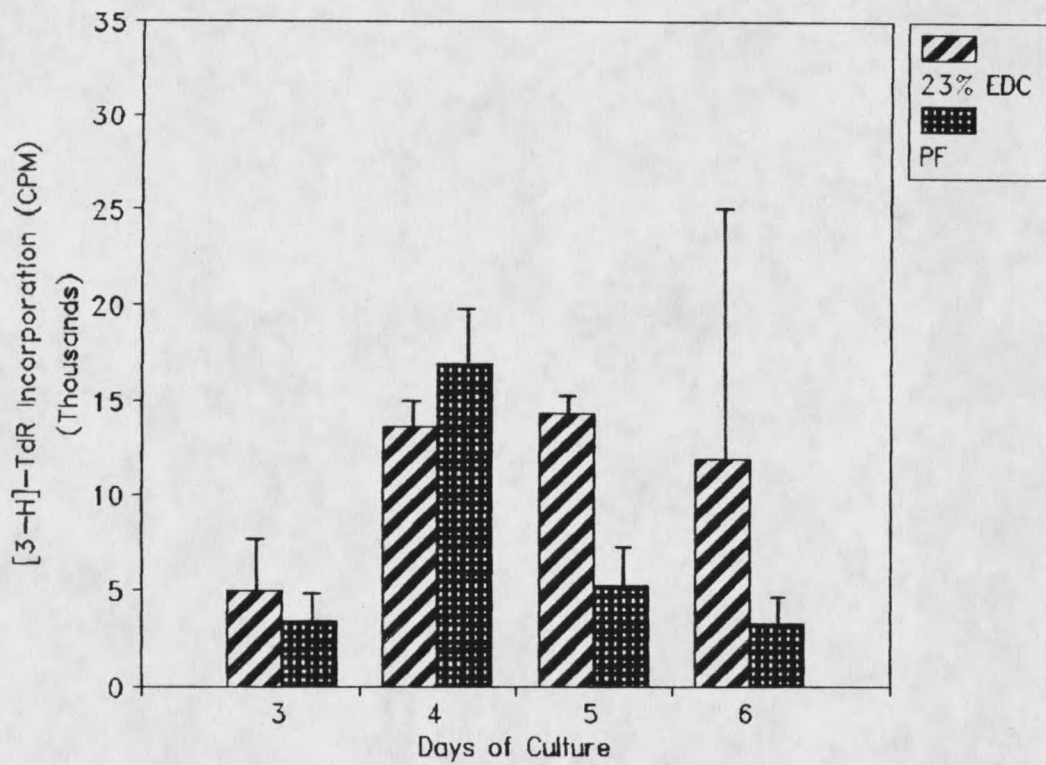


Figure 4. Syngeneic mixed lymphocyte reaction measured after 3, 4, 5 and 6 days culture. Proliferation response in SMLR of nylon-wool separated non-adherent ( $1 \times 10^6$  cells/well) from C57BL/6J spleen stimulated with mitomycin C inactivated nylon-wool separated adherent cells ( $5 \times 10^5$  cells/well) from the same animals.

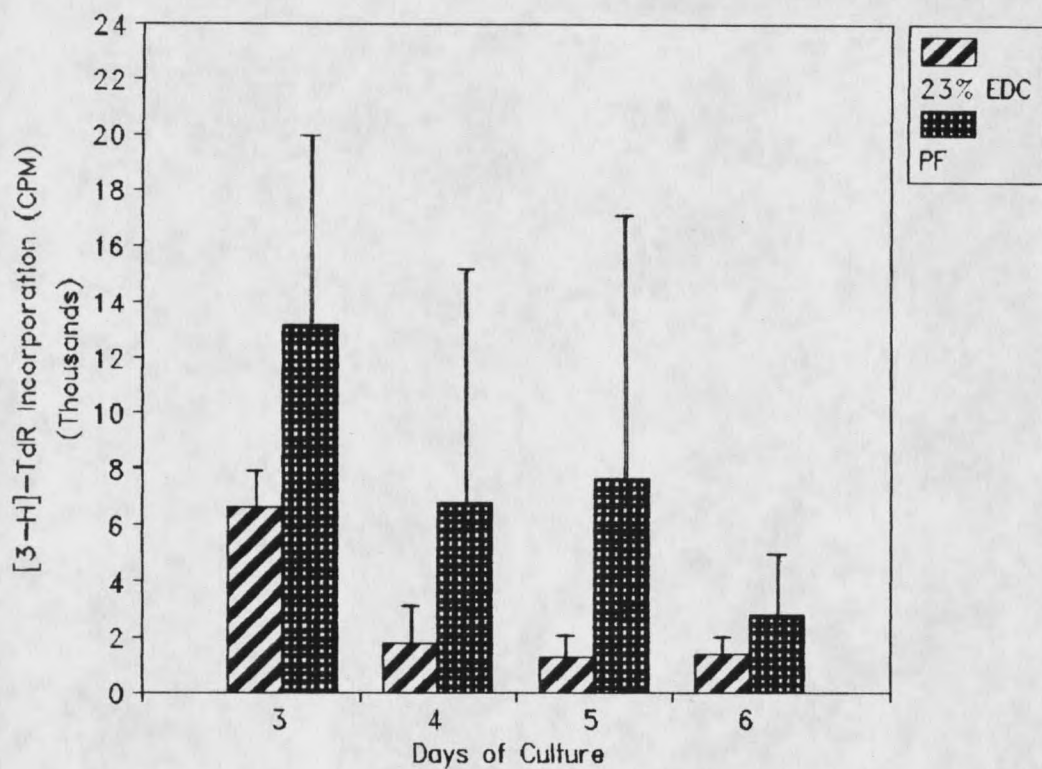


Figure 5. Syngeneic mixed lymphocyte reaction measured after 3, 4, 5 and 6 days culture. Proliferation response in SMLR of nylon-wool separated non-adherent ( $1 \times 10^6$  cells/well) from C57BL/6J spleen spleen stimulated with mitomycin C inactivated nylon-wool separated adherent cells ( $5 \times 10^5$  cells/well) from the same animals.

### Cytotoxic T Lymphocyte Responses

Another question we addressed was whether spleen cells of C57BL/6 young adult mice exposed to alcohol prenatally could normally generate cytotoxic lymphocytes. We used allogeneic mixed lymphocyte culture to generate cytotoxic T cells which were then used in a chromium release assay on P815 (H-2<sup>d</sup>) target cells to compare the cytotoxicity activity of 23% EDC and PF offspring. Table 10 shows lytic units (LU) per spleen and LU per 10<sup>6</sup> cells for CTL killing activity of both groups. In the first assay, the 23% EDC group had significantly higher cytotoxicity activity than PF group; on the contrary, in the second experiment, the PF group had higher killing activity than 23% EDC, although the difference was not statistically significant. But in the third experiment, the two groups showed very similar results for both LU and LU/10<sup>6</sup> cells. In all three CTL assays, the total recovery of viable cells was not significantly different between ethanol treated and control groups.

### RCS Driven Proliferation of Con-A Blasts

In this assay, we used spleen cells from 6-8 week old mice. A T-cell enriched population was obtained by nylon wool separation of spleen cells from both 23% EDC and PF groups to assay proliferation of Con A-blasts in RCS. As shown in Figure 6 and Figure 7, both groups had a similar pattern of

[<sup>3</sup>H]-thymidine incorporation in T lymphocytes pre-treated with Con A and then cultured with rat Con-A supernatant.

Table 10. Generation of CTL activity in 4 day allogeneic mixed lymphocyte culture.

Exp. No.	Diet Group	LU <sup>a</sup>	LU	Recovery of
		Culture	10 <sup>6</sup> Cells	Viable Cells (x 10 <sup>6</sup> )
		(±SD)	(±SD)	(±SD)
1	23% EDC <sup>b</sup>	49.0 <sup>c</sup> (±13.7)	16.4 (±5.1)	3.1 (±0.8)
1	PF <sup>d</sup>	30.8 (±4.4)	12.6 (±4.6)	2.7 (±0.9)
2	23% EDC	54.3 (±23.8)	23.1 (±12.3)	2.5 (±0.6)
2	PF	94.1 (±38.1)	42.7 (±20.6)	2.3 (±0.6)
3	23% EDC	127.9 (±22.3)	31.9 (±7.6)	4.1 (±0.5)
3	PF	129.0 (±53.6)	31.6 (±11.1)	4.0 (±0.4)

- a. LU, lytic unit, arbitrarily defined as cytotoxic activity required to achieve 50% specific lysis of target cells.  
 b. 23% EDC, 23% ethanol derived calories  
 c. Differs from PF at P < 0.05 level  
 d. PF, pair-fed against 23% EDC

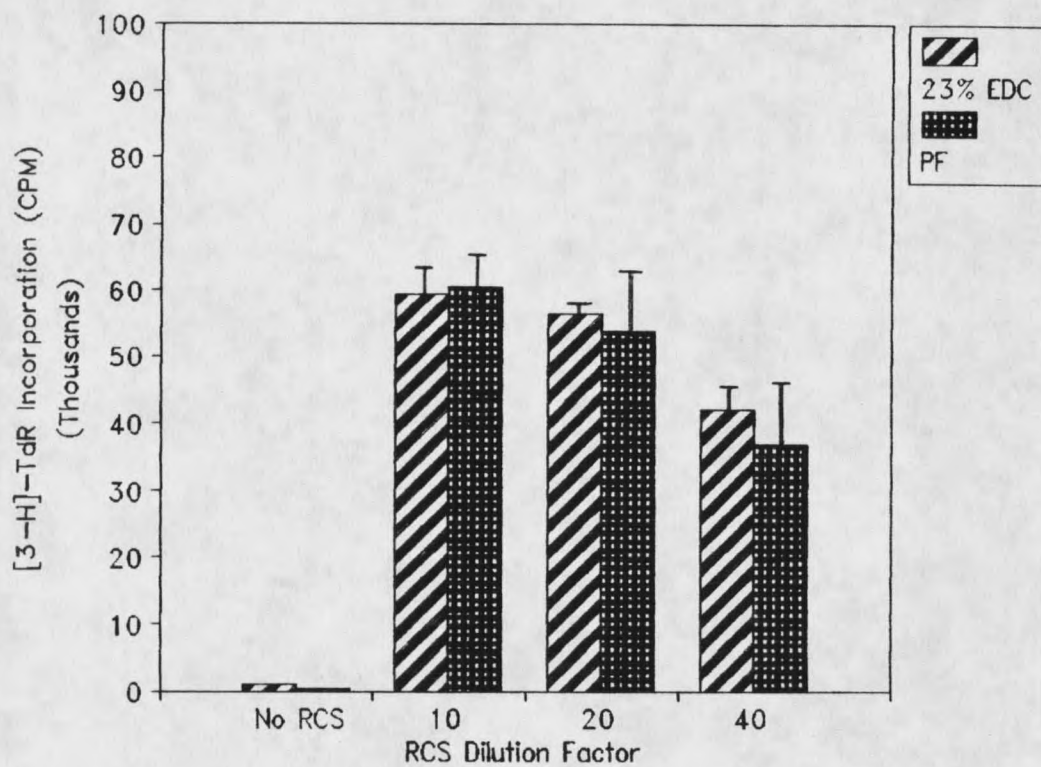


Figure 6. Nylon wool column enriched T lymphocytes ( $2 \times 10^7$  cells) were cultured with Con-A ( $2 \mu\text{g}/\text{ml}$ ) for 3 days. RCS (rat Con-A supernatant that contains IL-2) diluted 1:10, 1:20 or 1:40 was added to  $5 \times 10^4$  Con-A blast T cells for 24 hr.

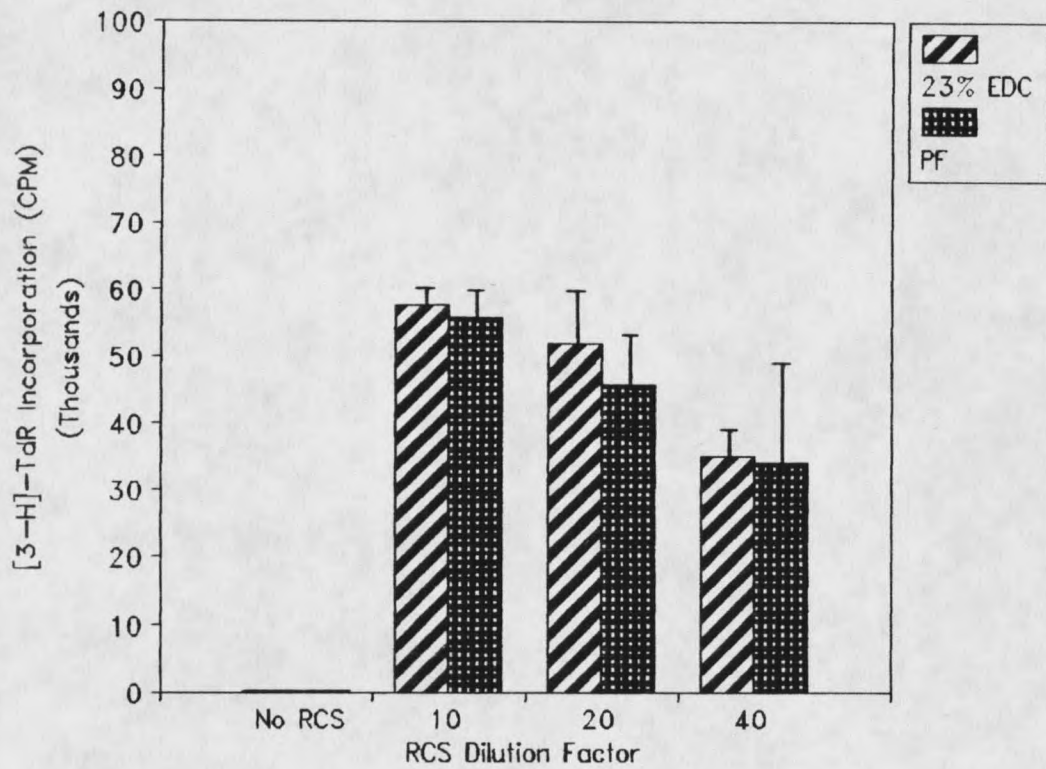


Figure 7. Nylon wool column enriched T lymphocytes ( $2 \times 10^7$  cells) were cultured with Con-A ( $2 \mu\text{g}/\text{ml}$ ) for 3 days. RCS (rat Con-A supernatant that contains IL-2) diluted 1:10, 1:20 or 1:40 was added to  $5 \times 10^4$  Con-A blast T cells for 24 hr.

## DISCUSSION

The first part of the study focused on a model involving acute ethanol exposure in utero, because we wished to test the hypothesis that prenatal acute ethanol in teratogenic doses would cause thymus aplasia or small size of thymus (DiGeorge Syndrome in human). As seen in the results, we found fetal limb malformation, missing fourth and/or fifth digits of the right forelimb in the fetuses of mice exposed to alcohol on gestation day 9 plus 12 hr and 16 hr. Similar results were also reported by Webster et al. (91,92) in a B6 mouse model of classical FAS. Additionally, 18-day fetuses exposed to ethanol on only one gestational day had thymuses that were significantly smaller than those of saline exposure groups, but thymocyte subpopulations defined by antigenic phenotype were not significantly different. Lighter weight fetuses (<0.8 g fetal weight) had even smaller thymuses, especially in the day 8 exposure group. No missing thymus was noted in any fetuses. We also found in this study that dams injected with ethanol on day 10 or 11 showed a high rate of resorption and lower number of fetuses; also fetal thymuses had reduced expression of differentiation antigens and cell number on day 18 of gestation.

We concluded that during thymus formation in B6 mouse, prenatal exposure to high doses of alcohol on one gestational day could result in a smaller than normal thymus, in addition

to previously described limb defects, low body weight and resorption. However, neither thymus stroma nor progenitor T cells from fetal liver and yolk sac were so severely or permanently affected by acute ethanol exposure on one gestational day as to lead to athymia. Perhaps a longer exposure to high dose of ethanol could lead to athymia in mice. It is also possible that genetic factors predispose to DiGeorge syndrome, and interact with ethanol or other teratogens.

It is also possible that further immune parameter examination of these alcohol-exposed pups after birth might demonstrate long term effects on immune function.

The second part of the study focused on the longer term immune consequences of chronic alcohol exposure in utero. Earlier studies from this laboratory showed that chronic exposure to ethanol throughout gestation led to thymuses in 18-day fetuses that were much smaller and less differentiated than normal. A logical question following from this observation was whether this abnormality would persist after birth. Therefore, examination of young adult animals with chronic prenatal alcohol exposure in immunologic assays could allow us to inspect both T and B cell compartments in more detail. A mouse model in which pregnant B6 mice are fed a liquid diet containing ethanol was used in our laboratory for several series of experiments and also was used in other laboratories as well (37). It is a very useful approach for



studying the effects of ethanol exposure in utero on immune function, because the well formulated liquid diets provide enough nutrition to eliminate other variables, such as malnutrition and liver dysfunction (68).

The results of this part of the study showed that average diet consumption, maternal weight gains and litter size were not significantly different for 23% ethanol derived calories (EDC) and pair-fed (PF) groups. In other words, there was nutritional equivalence between ethanol exposed and control groups. The reason we used 23% EDC instead of 25% EDC was because we planned to investigate immune function parameters of six to ten week old offspring. Based on our previous experience with alcohol diet feeding to C57BL/6 mice, slightly lower alcohol content could give a higher number of pups surviving for subsequent immune tests. After this feeding schedule, we examined several immune parameters using both in vivo and in vitro assays.

In the plaque forming cell (PFC) assay, our data demonstrated normal response to both thymus-dependent antigens and thymus independent antigens in young adult mice prenatally exposed to ethanol. Zidell et al. (93) using a CD-1 mouse model with intubation of 0, 6 and 9 g ethanol/kg body weight /day ethanol twice per day from gestation day 5 to gestation day 17, demonstrated that the PFC primary immune response to SRBC in the ethanol experimental group and control pair-fed groups were not significantly different. They did not

determine blood alcohol levels of these alcohol intubated mice, but cited work of others using their method who obtained BAL of 200 mg/100 ml. They also reported their mice showed profound central nervous system depression (indicating high BAL) after intubation. Although they claimed prenatal ethanol exposure induced some level of developmental delays and possible kidney pathology, they found no characteristic pattern of immune alteration in terms of primary PFC response to SRBC and delayed type hypersensitivity reaction to KLH.

In this study, cell-mediated immune responses were also examined in vivo and in vitro. The tests included delayed type hypersensitivity (DTH), contact sensitivity (CS), cytotoxic T lymphocyte (CTL) activity, AMLR, SMLR and RCS driven proliferation of Con-A blasts. The results in my study showed no significant difference between 23% EDC and PF groups in DTH, CS or proliferation of Con-A blasts to RCS, and no consistent differences in CTL assay. Zidell et al. (83) found no significant difference between EDC and PF group in the DTH response to KLH. On the other hand, Gottesfeld et al. (37) reported that offspring of C57BL/6 mice fed a 25% EDC diet from gestational day 0 to gestational day 20 showed depressed ability to produce cellular immune response as measured by contact sensitivity and a local graft-vs-host response. In their results, alcohol consumption was  $7.6 \pm 0.2$  g/kg/day and BAL was 30 mg/100 ml which were much lower than the alcohol

consumption of 20 g/kg/day (on g.d. 19) and BAL of 200 mg/100 ml of our experiment.

The only obvious difference between the methods of Gottesfeld and our laboratory was the ethanol content in liquid diet used: we used 23% EDC instead of their 25% EDC diet and the exposure period was from gestation day 5 to gestation day 19 in our study. One would predict that Gottesfeld's diet would produce higher ethanol consumption and higher BAL than ours. We can not reconcile their low ethanol consumption with our results with this diet regimen. They also claimed that altered noradrenergic synaptic transmission selectively in lymphoid organs might cause change in immune function in the fetal alcohol syndrome. Our results suggested that under 23% EDC feeding, there was no alteration of DTH, CS, CTL and proliferation of Con-A blast T cell responses.

The first animal FAS model reporting immune changes in adult animals was by Monjan and Mandell (64) in 1980. They documented depressed mitogen-induced lymphocyte blastogenesis in T lymphocytes. They used a rat model and intubated diet with 10% to 30% of ethanol which resulted in maternal ethanol consumption of 1, 3, or 6 g/kg/day; this level was lower than ours (20 g/kg ethanol consumption on gestational day 19 of 23% EDC feeding regimen) in B6 mouse model. They also reported that B lymphocyte function was normal in LPS induced blastogenesis. Their results were based on a small number of rats. Norman et al. (66) used rats in FAS model with 5% (w/v)

ethanol (equivalent to 36% EDC) diet feeding from gestational day 8 to gestational day 20, resulting in ethanol consumption of 10.9 g/kg/day. They found that three month old offspring of the alcohol treated group had normal Con-A responses but diminished T cell responses in RCS or IL-2 driven proliferation of Con-A blast T cells. Redei et al. (74) also examined immune function in offspring of rats fed 35% EDC diet from gestation day 15 to parturition (10.2 g/kg/day of maternal ethanol consumption). Those authors reported depressed spleen T cell responses of three week old offspring from alcohol treated group in Con-A induced proliferation assay.

Recently, Rao et al. (72) reviewed the 36% EDC diet used in the FAS rat model and claimed that it produced malnutrition during gestation. Pregnant rats in the 36% EDC experimental group consumed 56 ml per day which was considerably less than the isocaloric control group given diet ad-libitum, which drank 90 ml/day. In addition, the 36% EDC diet contains only 11% carbohydrate compared to 47% in the control diet. In other words, the 36% EDC diet is deficient in carbohydrate which is very important during embryo development. On our 23% EDC diet, mice drank almost as much as control mice given diet ad-libitum and the 23% EDC formulation contains adequate carbohydrates. Hence, deleterious effects of prenatal alcohol exposure on the immune system reported by some groups may be due to a combination of inadequate nutrition and alcohol.

Taken together, the diet composition, food intake and different species may be causing the different results reported in chronic prenatal alcohol exposure experiments. Higher ethanol concentration in the 36% EDC diet could cause the animal to eat less food, leading to nutritional problems that then cause immune alterations in the rat FAS model. It is well-known that malnutrition in the prenatal period can have long lasting effects on immune response (28).

We did additional in vitro T lymphocyte function tests, such as allogeneic mixed lymphocyte reaction (AMLR) and syngeneic mixed lymphocyte reaction (SMLR), on adult animals exposed to alcohol in utero. Both AMLR and SMLR are antigen-induced proliferative responses that are used to study the genetic and cellular requirement for immune induction, especially for testing T cell function. SMLR is used to measure a cellular interaction between T cell subset and I-a antigen on the macrophage like cells. The reaction may have physiological importance in the regulation of T cell function. Therefore, testing SMLR would detect the effects of prenatal alcohol exposure to T cell immune function. Both of these reactions have been studied in several species, including humans, mice and rats. The cell proliferation pattern of 23% EDC and PF group pups showed no significant differences in either AMLR (except in the first AMLR on day 3) or SMLR assay. Therefore, 23% EDC feeding to pregnant dams did not cause

obvious T cell function alteration in spleen T cells of their young adult offspring.

Because the thymus is a regenerating system, damage to the lymphoid compartment in fetal life might recover after 4 to 8 weeks of good nourishment after birth. Data showed that although 18-day fetuses from 23% EDC diet group have small and immature thymuses (Ewald, unpublished data) at the age of 4 to 8 weeks mice have recovered as assessed by a variety of immunological tests. One of the questions we had was whether thymus damage seen in 18-day fetuses on chronic diet was in epithelial compartment or lymphoid. The possibility of injury to different parts of the thymus might have different consequences to the immune system in adulthood. Damage to the lymphoid compartment would likely regenerate after birth given adequate nutrition, which may account for the results in this B6 chronic alcohol exposure experiment. These young pups with good nourishment have recovered from minor thymus damage. On the contrary, in human DiGeorge syndrome, damage to fetal thymus stroma does not recover after birth. Thymus aplasia or small thymus, and T cell immune alterations thus persist after birth.

Johnson et al. (45) reported that humans with FAS had immune deficiency due to intrauterine exposure to alcohol. In contrast, our mouse FAS model did not have the same results. Poor nutrition is common among human alcoholics as is use of other drugs. These variables complicate the interpretation of

immune alterations in children with FAS. Also, murine thymus development differs from human thymus development in degree of maturation occurring prenatally. Therefore, the susceptibility to alcohol during thymus ontogeny may vary between human and mouse. Especially, in postnatal period, with good nutrition, prenatal ethanol exposure may not result in immune dysfunction in the young adult C57BL/6 mouse.

In summary, the results suggest that acute prenatal exposure to high doses of alcohol does not induce thymus aplasia. Furthermore, chronic alcohol exposure in the prenatal period does not result in persistent immune alterations in the B6 mouse. However, the immune system is a complex system that is interconnected with other organs and systems such as the hormone and neural systems. In the present study, we examined only T cell and B cell immune function and thymus organ development. Other immune assays, such as NK cell activity, lymphokine secretion, macrophage function (antigen presenting function) and neural-immune connections remain to be investigated.

## REFERENCES CITED

1. Abdallah, R.M., J.R. Starkey, and G.G. Meadows. 1988. Toxicity of chronic high alcohol intake on mouse natural killer cell activity. *Res. Commun. Chem. Pathol. Pharmacol.* 59:245.
2. Abdallah, R.M., J.R. Starkey, and G.G. Meadows. 1983. Alcohol and related dietary effects on mouse natural killer-cell activity. *Immunology* 50:131.
3. Adkins, B., C. Muller, C.Y. Okada, R.A. Reichert, I.L. Weissman and G.J. Spangrude. 1987. Early events in T-cell maturation. *Ann. Rev. Immunol.* 5:325.
4. Aldo-Benson, M. 1989. Mechanisms of alcohol-induced suppression of B-cell response. *Alcohol Clin. Exp. Res.* 13:469.
5. Ammann, A.J., D.W. Wara, M.J. Cowan, D.J. Barrett, and E. R. Stiehm. 1982. The DiGeorge syndrome and the fetal alcohol syndrome. *Am J Dis Child* 136:906.
6. Angelogianni, P. and C. Gianoulakis. 1989. Prenatal exposure to ethanol alters the ontogeny of the endorphin response to stress. *Alcohol Clin. Exp. Res.* 13:564.
7. Bagasra, O., A. Howedy, R. Dorio, and A. Kajdacsy-Balla. 1987. Functional analysis of T-cell subsets in chronic experimental alcoholism. *Immunology* 61:63.
8. Barron, S., S.B. Tieman, and E.P. Riley. 1988. Effects of prenatal alcohol exposure on the sexually dimorphic nucleus of the preoptic area of the hypothalamus in male and female rats. *Alcohol Clin. Exp. Res.* 12:59.
9. Baruah, J.K. and D. Kinder. 1989. Pathological changes in peripheral nerves in experimental fetal alcohol syndrome. *Alcohol Clin. Exp. Res.* 13:547.
10. Blume, S.B. 1986. Women and alcohol. *JAMA* 256:1467.
11. Boggan, W.O., C.L. Randall, and H.M. Dodds. 1979. Delayed sexual maturation in female C57BL/6J mice prenatally exposed to alcohol. *Res. Commun. Chem. Pathol. Pharmacol.* 23:117.
12. Brayton, R.G., P.E. Stokes, M.S. Schwartz, and D.B. Louria. 1970. Effect of alcohol and various diseases on leukocyte mobilization, phagocytosis and intracellular bacterial killing. *N. Eng. J. Med.* 282:123.



13. Brown, N.A., E.H. Goulding, S. Fabro. 1979. Ethanol embryotoxicity: Direct effects on mammalian embryos in vitro. *Science* 206:573.
14. Brunner, K.T., J. Mael, J-C. Cerottini, and B. Chapuis. 1968. *Immunology* 14:181.
15. Charpentier, B., D. Franco, L. Paci, M. Charra, B. Martin, D. Vuitton, and D. Fries. 1984. Deficient natural killer cell activity in alcoholic cirrhosis. *Clin. Exp. Immunol.* 58:107.
16. Chen, J.J., and E.R. Smith. 1979. Effects of prenatal alcohol on sexual differentiation and open-field behavior in rats. *Horm. Behav.* 13:219.
17. Chernoff, G.F. 1980. The fetal alcohol syndrome in mice: maternal variables. *Teratology* 22:71.
18. Clarren, S.K. and D.W. Smith. 1978. The fetal alcohol syndrome. *N. Engl. J. Med.* 298:1063.
19. Clarren, S.K. 1981. Recognition of fetal alcohol syndrome. *JAMA* 245:2436.
20. Clarren, S.K., E.C. Alvord, S.M. Sumi, A.P. Streissguth, and D.W. Smith. 1978. Brain malformations related to prenatal exposure to ethanol. *J. Pediatr.* 92:64.
21. Criteria Committee, National Council On Alcoholism, New York, New York. 1972. Criteria for the diagnosis of alcoholism. *Ann. Intern. Med.* 77:249.
22. Eckardt, M.J., T.C. Harford, C.T. Kaelber, E.S. Parker, L.S. Rosenthal, R.S. Ryback, G.C. Salmoiraghi, E. Vanderveen, and K.R. Warren. 1981. Health hazards associated with alcohol consumption. *JAMA* 246:648.
23. Daft, P.A., M.C. Johnston, and K. K. Sulik. 1986. Abnormal heart and great vessel development following acute ethanol exposure in mice. *Teratology* 33:93.
24. Diaz, J. and H.H. Samson. 1980. Impaired brain growth in neonatal rats exposed to ethanol. *Science* 208:751.
25. Ewald, S.J. and W.W. Frost. 1987. Effect of prenatal exposure to ethanol on development of the thymus. *Thymus* 9:211.
26. Ewald, S.J. 1989. T Lymphocyte populations in fetal alcohol syndrome. *Alcohol Clin. Exp. Res.* 13:485.

27. Ewald, S.J. and S.M. Walden. 1988. Flow cytometric and histological analysis of mouse thymus in fetal alcohol syndrome. *J. Leukocyte Biol.* 44:434.
28. Ferguson, A.C., G.L. Lawlor, C.G. Neumann, W. Oh. and E.R. Stiehm. 1974 Decreased rosette-forming lymphocyte in malnutrition and intrauterine growth. *J. Pediatr.* 85:717.
29. Fitch, F.W., H.D. Engers, H.R. MacDonald, J-C. Cerottini, and K.T. Brunner. 1975. Generation of cytotoxic T lymphocyte in vitro VI. effects of cell density on response in mixed leukocyte cultures. 1975. *J. Immunol.* 115:1688.
30. Fowlkes, B.J., R.H. Schwartz and D.M. Pardoll. 1988. Deletion of self-reactive thymocytes occurs at a CD4<sup>+</sup>CD8<sup>+</sup> precursor stage. *Nature* 334:620.
31. Friedman, J. M. 1982. Can maternal alcohol ingestion cause neural tube defects? *J. Pediatr.* 101:232.
32. Gonzales, E.R. 1981. New ophthalmic findings in fetal alcohol syndrome. *JAMA* 245:108.
33. Gluckman, S.J., V.C. Dvorak, and R.R. MacGregor. 1977. Host defenses during prolonged alcohol consumption in a controlled environment. *Arch Intern Med* 137:1539.
34. Golden, N.L., R.J. Sokol, B.R. Kuhnert, and S. Bottoms. 1982. Maternal alcohol use and infant development. *Pediatrics* 70:931.
35. Gonzales, R.A., C. Theiss, and F.T. Crews. 1986. Effects of ethanol on stimulated inositol phospholipid hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.* 237:92.
36. Gottesfeld, Z., B. Morgan, and J.R. Perez-Polo. 1990. Prenatal alcohol exposure alters the development of sympathetic synaptic components and of nerve growth factor receptor expression selectively in lymphoid organs. *J. Neurosci.* 35:185.
37. Gottesfeld, Z., R. Christie, D.L. Felten, and S.J. LeGrue. 1990. Prenatal ethanol exposure alters immune capacity and noradrenergic synaptic transmission in lymphoid organs of the adult mouse. *J. Neurosci* (in press).
38. Grossman, C.J., C.L. Mendenhall, and G.A. Roselle. 1988. Effects of ethanol on concanavalin a sensitive thymic lymphocyte. *Int. J. Immunopharmac.* 10:187.

39. Ha, T-Y., N.D. Reed, and P.K. Crowle. 1986. Immune response potential of mast cell-deficient W/W mice. *Int. Archs Allergy Appl. Immun.* 80:85.
40. Hanson, J.W., K.L. Jones, and D.W. Smith. 1976. Fetal alcohol syndrome. *JAMA* 235:1458.
41. Harp, J.A., B.S. Davis, and S.J. Ewald. 1984. Inhibition of T cell responses to alloantigens and polyclonal mitogens by Ly-5 antisera. *J. Immunol.* 133:10.
42. Hingson, R., J.J. Alpert, N. Day, E. Dooling, H. Kayne, S. Morlock, E. Oppenheimer, and B. Zuckerman. 1982. Effects of maternal drinking and marijuana use on fetal growth and development. *Pediatrics* 70:539.
43. Jenkins, J.S. and J. Connolly. 1968. Adrenocortical response to ethanol in man. *Br. Med. J.* 2:804.
44. Jerrells, T.R., D. Peritt, C. Marietta, and M.J. Eckardt. 1989. Mechanisms of suppression of cellular immunity induced by ethanol. *Alcohol Clin. Exp. Res.* 13:490.
45. Johnson, S., R. Knight, D.J. Marmor, and R. W. Steele. 1981. Immune deficiency in fetal alcohol syndrome. *Pediatr. Res.* 15:908.
46. Jones, K.L., D.W. Smith, C.N. Ulleland, and A.P. Streissguth. 1973. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* 1:1267.
47. Jones, K.L. and D.W. Smith. 1973. Recognition of the fetal alcohol syndrome in early infancy. *Lancet* 2:99.
48. Kalter, H., J. Warkany. 1983. Congenital malformations. *N. Engl. J. Med.* 308:491.
49. Kaplan, D.R. 1986. A novel mechanism of immunosuppression mediated by ethanol. *Cell. Immunol.* 102:1.
50. Kincade, P.W. 1981. Formation of B lymphocyte in fetal and adult life. *Adv. Immunology* 31:177.
51. Lemoine P., H. Hauousseau, J.P. Borteyru and J.C. Menuet. 1968. Les enfants de parents alcooliques: anomalies observers. A propos de 127 cas. *Quest Med.* 21:476.
52. Lieber, C.S. 1975. Interference of ethanol in hepatic cellular metabolism. *Ann. N.Y. Acad. Sci.* 252:24.

53. Lieber, C.S., and DeCarli, L.M. 1982. The feeding of alcohol in liquid diets: Two decades of applications and 1982 update. *Alcohol Clin. Exp. Res.* 6:532.
54. Lobach, D.F. and B.F. Haynes. 1987. Ontogeny of the human thymus fetal development. *J. Clin. Immunol.* 7:81.
55. MacGregor, R.R., S.J. Gluckman, and J.R. Senior. 1978. Granulocyte function and levels of immunoglobulin and complement in patients admitted for withdrawal from alcohol. *J. Infect. Dis.* 138:747.
56. MacGregor, R.R. 1986. Alcohol and immune defense. *JAMA* 256:1474.
57. Majchrowicz, E. 1975. Induction of physical dependence upon ethanol and the associated behavioral changes in rats. *Psychopharmacologia* 43:245.
58. Meadows, G.G., S.E. Blank, and D.D. Duncan. 1989. Influence of ethanol consumption on natural killer cell activity in mice. *Alcohol Clin. Exp. Res.* 13:476.
59. Mekori, Y.A., J.C.C. Chang, B.K. Wershil, and S.J. Galli. 1987. Studies of the role of mast cells in contact sensitivity responses. *Cell. Immunol.* 109:39.
60. Mendelson, J.H., N.K. Mello. 1979. Biologic concomitant of alcoholism. *N. Eng. J. Med.* 301:912.
61. Mishell, R.I., R.W. Dutton. 1967. Immunization of dissociated spleen cell culture from normal mice. *J. Exp. Med.* 126:423
62. Mishell, B.B., S.M. Shiigi. 1980. In *Selected Methods in Cellular Immunology*. pp. 182-185. Freeman, San Francisco CA.
63. Mochly-Rosen, D., F-H. Chang, L. Cheever, M. Kim, I. Diamond, and A.S. Gordon. 1988. Chronic ethanol causes heterologous desensitization of receptors by reducing messenger RNA. *Nature* 333:848.
64. Monjan, A.A. and W. Mandell. 1980. Fetal alcohol and immunity: Depression of mitogen-induced lymphocyte blastogenesis. *Neurobehav. Toxicol. Teratol.* 2:213.
65. Mukherjee A.B. and G.D. Hodgen. 1982. Maternal ethanol exposure induces transient impairment of umbilical circulation and fetal hypoxia in monkeys. *Science* 218:700.

66. Norman, D.C., M-P. Chang, S.C. Castle, J.E. Van Zuylen, and A.N. Taylor. 1989. Diminished proliferative response of Con A-blast cells to interleukin 2 in adult rats exposed to ethanol in utero. *Alcohol Clin. Exp. Res.* 13:69.
67. Ouellette, E.M., H.L. Rosett, N.P. Rosman, and L. Weiner. 1977. Adverse effects on offspring of maternal alcohol abuse during pregnancy. *N. Eng. J. Med.* 297:528.
68. Osmond, D.G. 1980. B lymphocyte genesis in the bone marrow. *Prog. Immunol.* 6:49.
69. Pennington, S. 1988. Ethanol-induced growth inhibition: The role of cyclic AMP-dependent protein kinase. *Alcohol Clin. Exp. Res.* 12:125.
70. Plant, M.L. and M.A. Plant. 1988. Maternal use of alcohol and other drugs during pregnancy and birth abnormalities: further results from a prospective study. *Alcohol & Alcohol.* 23:229.
71. Randall, C.L. and W.J. Taylor. 1979. Prenatal ethanol exposure in mice : teratogenic effects. *Teratology* 19:305.
72. Rao, G.A., E.C. Larkin, and R.F. Derr. 1988. Chronic alcohol consumption during pregnancy: alleviation of untoward effects by adequate nutrition. *Nutr. Res.* 8:421.68.
73. Rasmussen, B.B. and N. Christensen. 1980. Teratogenic effects of maternal alcohol consumption on the mouse fetus. *ACTA Path. Microbiol. Scand Sect. A.* 88:2285.
74. Redei, E., W.R. Clark, and R.F. McGivern. 1988. Alterations in immune responsiveness ACTH and CRF content of brain in animals exposed to alcohol during the last week of gestation. *Alcohol Clin. Exp. Res.* 13:439.
75. Roselle, G.A. and C.L. Mendenhall. 1984. Ethanol-induced alterations in lymphocyte function in the guinea pig. *Alcohol Clin. Exp. Res.* 8:62.
76. Rosett, H.L., L. Weiner, and K.C. Edelin. 1983. Treatment experience with pregnant problem drinkers. *JAMA* 249:2029.
77. Rosett, H.L. and L. Weiner. 1985. Alcohol and pregnancy: a clinical perspective. *Ann. Rev. Med.* 36:73.

78. Royo, C., J.L. Touraine, and O. Bouteiller. 1987. Ontogeny of T lymphocyte differentiation in the human fetus: acquisition of phenotype and functions. *Thymus* 10:57-73.
79. Sandor, George G.S., D.F. Smith, and P.M. MacLeod. 1981. Cardiac malformations in the fetal alcohol syndrome. *J. Pediatr.* 98:771.
80. Sheppard, J., P. Alberstein and B. McClearn. 1968. Enzyme activities and ethanol preference in mice. *Biochem Genet.* 2:205.
81. Sokol, R.J. and S.K. Clarren. 1989. Guidelines for use of terminology describing the impact of prenatal alcohol on the offspring. *Alcohol Clin. Exp. Res.* 13:597.
82. Spagnuolo, P.J. and R.R. MacGregor. 1974. Acute ethanol effect on chemotaxis and other components of host defense. *J. Lab. Clin. Med.* 86:24.
83. Steinhausen, H.C., V. Nestler, and H. Huth. 1982. Psychopathology and mental functions in the offspring of alcoholic and epileptic mothers. *J. Am. Acad. Child Psychiatry* 3:268.
84. Streissguth, A.P., S.L. Dwyer, J.C. Martin, and D.W. Smith. 1980. Teratogenic effects of alcohol in humans and laboratory animals. *Science* 209:353.
85. Streissguth, A.P., C.S. Herman, and D.W. Smith. 1978. Intelligence, behavior, and dysmorphogenesis in the fetal alcohol syndrome: A report on 20 patients. *J. Pediatr.* 92:363.
86. Suzuki, R., S. Suzuki, M. Igarashi, and K. Kumagai. 1986. Induction of interleukin 3 but not interleukin 2 or interferon production in the syngeneic mixed lymphocyte reaction. *J. Immunol.* 137:1564.
87. Tennebaum, J.I., R.D. Ruppert, R.L. St.Pierre, and N.J. Greenberger. 1969. The effect of chronic alcohol administration on the immune responsiveness of rats. *J. Allergy* 44:272.
88. Walia A.S., E.W. Lamon. 1990. In vitro effects of ethanol and acetaldehyde on cell-mediated cytotoxicity: Alcohol, Immunomodulation and AIDS. pp.145-153. Alan R. Liss Inc., NY.

89. Watson, R.R., J.C. Jackson, B. Hartmann, R. Sampliner, D. Mobley, RN, and C. Eskelson. 1985. Cellular immune functions, endorphins, and alcohol consumption in males. *Alcohol Clin. Exp. Res.* 9:248.
90. Webster, W.S., M-S. Germain, A. Lipson, and D. Walsh. 1984. Alcohol and congenital heart defects: an experimental study in mice. *Cardiovasc. Res.* 18:335.
91. Webster, W.S., D.A. Walsh, S.E. McEwen, and A.H. Lipson. 1983. Some teratogenic properties of ethanol and acetaldehyde in C57BL/6J mice: implications for the study of the fetal alcohol syndrome. *Teratology* 27:231.
92. Wharton, R.S., T.S. Sievenpiper, and R.I. Mazze, 1980. Developmental toxicity of methoxyflurane in mice. *Aneth. Analg.* 59:42.
93. Zidell, R.H., N.S. Hatoum, and P.T. Thomas. 1988. Fetal alcohol effects: Evidence of developmental impairment in the absence of immunotoxicity. *Fundamental and Applied Toxicology* 10:189.

MONTANA STATE UNIVERSITY LIBRARIES



3 1762 10075086 6

