The C3H/HeJ mouse in vivo and in vitro antibody response to endotoxin
by Craig William Spellman

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
Montana State University

Abstract:
The C3H/HeJ mouse strain has been investigated recently because it exhibits an anomalous response to endotoxin from gram negative bacteria. This peculiar response has enabled further investigations into the . mechanisms of B cell activation. This paper examines the in vivo and in vitro antibody response by this mouse strain because there is a paucity of data in this area, and because the explanation for the anomalous response itself has remained open. It was demonstrated that the C3H/HeJ animals could make primary and secondary responses to the thymus-dependent antigen sheep erythrocytes in vivo and in vitro. Primary in vivo responses were also demonstrated for the thymus-independent antigens Vi, Vi-positive Citrobacter freundil bacterin, polyvinylpyrrolidone, and native protoplasmic polysaccharide. This mouse strain did not make primary or secondary responses to Escherichia coli 0113 purified lipopolysaccharide either in vivo or in vitro. In vivo primary, but not secondary, anti-lipopolysaccharide responses by C3H/HeJ spleen cells to E. coli bacterin could be demonstrated, whereas in vitro, strong secondary anti—lipopolysaccharide responses to the E. coli bacterin could be elicited. A primary in vitro response to the E. coli bacterin by C3H/HeJ spleen cells is also suggested from a conservative interpretation of the data. Cell cultures from C3H/HeJ mice yielded mitogenic responses to phytohemagglutinin, concanavalin A, and E. coli bacterin. Whether or not the C3H/HeJ strain cell cultures gave a mitogenic response to lipopolysaccharide could not be established. Hybrid F1 mice (Balb/c X C3H/HeJ), when tested, responded similarly to the Balb/c mice. The demonstration of specific anti-lipopolysaccharide antibody responses suggests that the low or absent C3H/HeJ lipopolysaccharide response noted by other investigators may result from the particular preparation of endotoxin used in assays.

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Date August 9, 1976

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by

CRAIG WILLIAM SPELLMAN

A thesis submitted in partial fulfillment
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ABBREVIATIONS

B cell	bursa-equivalent lymphocyte
Con A	concanavalin A
i.p.	intraperitoneal
i.v.	intravenous
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
LPS 0113	lipopolysaccharide from *E. coli* 0113
MLC	mixed lymphocyte culture
NPP	native protoplasmic polysaccharide
PBS	phosphate buffered saline
PFC	plaque forming cell
PHA	phytohemagglutinin
PVP	polyvinylpyrrolidone
R^{o}	residual response
SRBC	sheep red blood cells
SSS III	type III pneumococcal polysaccharide
TCA	trichloroacetic acid
T cell	thymus-derived cell
1^{o}	primary response or primary injection
2^{o}	secondary response or secondary injection

ABSTRACT

The C3H/HeJ mouse strain has been investigated recently because it exhibits an anomalous response to endotoxin from gram negative bacteria. This peculiar response has enabled further investigations into the mechanisms of B cell activation. This paper examines the *in vivo* and *in vitro* antibody response by this mouse strain because there is a paucity of data in this area, and because the explanation for the anomalous response itself has remained open. It was demonstrated that the C3H/HeJ animals could make primary and secondary responses to the thymus-dependent antigen sheep erythrocytes *in vivo* and *in vitro*. Primary *in vivo* responses were also demonstrated for the thymus-independent antigens Vi, Vi-positive *Citrobacter freundii* bacterin, polyvinylpyrrolidone, and native protoplasmic polysaccharide. This mouse strain did not make primary or secondary responses to *Escherichia coli* 0113 purified lipopolysaccharide either *in vivo* or *in vitro*. *In vivo* primary, but not secondary, anti-lipopolysaccharide responses by C3H/HeJ spleen cells to *E. coli* bacterin could be demonstrated, whereas *in vitro*, strong secondary anti-lipopolysaccharide responses to the *E. coli* bacterin could be elicited. A primary *in vitro* response to the *E. coli* bacterin by C3H/HeJ spleen cells is also suggested from a conservative interpretation of the data. Cell cultures from C3H/HeJ mice yielded mitogenic responses to phytohemagglutinin, concanavalin A, and *E. coli* bacterin. Whether or not the C3H/HeJ strain cell cultures gave a mitogenic response to lipopolysaccharide could not be established. Hybrid F_1 mice (Balb/c X C3H/HeJ), when tested, responded similarly to the Balb/c mice. The demonstration of specific anti-lipopolysaccharide antibody responses suggests that the low or absent C3H/HeJ lipopolysaccharide response noted by other investigators may result from the particular preparation of endotoxin used in assays.

INTRODUCTION

Lipopolysaccharide (LPS) isolated from gram negative bacteria can be viewed as a composite of two distinct functional moieties: The O-polysaccharide which has been characterized as the major antigen (38) is linked via a trisaccharide of 2-keto-3-deoxyoctanoic acid (KDO) to the lipid A structure (38).

LPS can elicit a myriad of biological activities from a host. Included in the list of host-reactive immunological properties are: 1) specific mitogenicity for B lymphocytes (B cells) (3,4,11,20,47), 2) highly immunogenic (35,54), 3) polyclonal activation of B cells to antibody producing cells (4,14), 4) replacement of thymus cell (T cell) helper function (57,69,70), 5) adjuvant activity which enhances the immune response to other antigens (1,23,32,33,59), 6) interference with the induction of tolerance to protein antigens (22,36,37), and 7) LPS has been demonstrated to be a thymus-independent (T-independent) antigen in that T cells are apparently not required for an immune response to LPS (5,39,43).

It has been difficult to formally relate these diverse immunologic activities of LPS to a common

mechanism; however recent experiments utilizing the C3H/HeJ strain of mice have provided a model, not dependent upon chemical modifications such as acid or base hydrolysis of LPS, for understanding the signals involved.

The C3H/HeJ mouse strain, developed at the L. C. Strong Laboratory (Del Mar, CA), was the original C3H strain from which several other sublines derived (26). The subline C3H/He subsequently yielded the C3H/HeN and C3H/HeJ strains which have been maintained separately since 1948 at the Jackson Laboratories (Bar Harbor, ME) (52). The C3H/HeJ mouse has been intensively investigated recently following the initial observations by Sultzer that this particular strain reacted in an anomalous manner to endotoxin (63). The C3H/HeJ mice were very resistant to endotoxin toxicity and exhibited a peculiar leukocyte response to intraperitoneal injections of Escherichia coli (E. coli) or Salmonella typhosa endotoxin in that the ratio of neutrophils to mononuclear cells was small compared to other mice strains (63). The C3H/HeJ mice have been demonstrated to be refractory to many other properties of LPS.

a) Mitogenic Response: The lipid A moiety of LPS was shown to be a B cell mitogen (11,20,47), but spleen cell cultures prepared from C3H/HeJ mice were not stimulated when tested with several types of E. coli or Salmonella spp. LPS (11,15,64,66,71,72). The response to other B cell mitogens, purified protein derivative from tuberculin (64), dextran sulfate, and polyinosinic acid (56), was normal in the C3H/HeJ mice indicating that the inability to respond to LPS did not limit other mitogenic responses (71).

b) Adjuvant Activity of LPS: It was observed that LPS could function as an adjuvant for protein antigens (1,23,33), and the lipid A portion was responsible for the adjuvant effect (11). This adjuvant effect could not be demonstrated in C3H/HeJ mice. Skidmore et al have shown that C3H/HeJ and A/J mice responded equally well to the antigen bovine serum albumin (BSA), but that in A/J mice LPS enhanced the BSA response more than 1000 fold while there was no enhancement with LPS in C3H/HeJ mice (59).

c) Inhibition of Tolerance: It has been reported by Claman (12), Golub et al (22), and Louis et al (36)

that LPS administered shortly after a tolerogenic dose of a protein antigen such as deaggregated human gamma-globulin inhibited the induction of tolerance. This phenomenon could not be demonstrated in C3H/HeJ mice (59). The failure of LPS to interfere with the induction of tolerance was not due to the inability of the strain to respond to the antigen because C3H/HeJ mice did respond to aggregated human gamma-globulin (59).

d) Polyclonal Response: An antibody response to many antigens, due to polyclonal B cell activation by LPS was reported by Coutinho et al (14). Experiments have shown that C3H/HeJ mice support only a very small polyclonal response as assayed by antibody specific for the hapten trinitrophenyl (26,71).

e) Immune Response: It has been clearly shown that C3H/HeJ mice were low responders to LPS over a wide dose range and that the optimum dose was between 1 and 25μg of LPS (59,71). Skidmore et al (59) reported that the immune response to LPS peaked on day 7 and diminished on days 9-13. Reed and Rudbach indicated that the peak response occurred on day 4

and diminished thereafter in C3H/HeJ animals (48). The kinetics of the response may depend upon which endotoxin preparation is used. C3H/HeJ mice did respond to native protoplasmic polysaccharide (NPP), an aberrant form of LPS which lacks lipid A and KDO but cross reacts completely with the O-antigenic determinants of homologous LPS (67), similar in magnitude to that observed in other high LPS responder C3H strains. This indicated that the low LPS response by C3H/HeJ mice to LPS was due to a failure by the C3H/HeJ mice to respond to the lipid A structure and not to the O-polysaccharide (73). Further, since the NPP responses were similar to the LPS responses in C3H/HeJ animals, the implication was that the lipid A did not function as an adjuvant for the O-polysaccharide antigen (67,73). Jacobs (26) reported that C3H/HeJ mice, which could make a response to trinitrophenyl-keyhole limpet hemocyanin, made a very low response to trinitrophenyl-LPS (a thymus-independent antigen) suggesting an active role for the immunogenicity of the carrier was dependent on lipid A.

To determine if the anomalous C3H/HeJ responses to LPS were due to a defect in one or multiple genes, Watson and Riblet performed a genetic analysis (71,72). It was demonstrated that the ability to be a high LPS responder was dominant because F_1 animals from high and low responder parents gave high LPS responder progeny. Mixed lymphocyte cultures (MLC) using C3H/HeJ and C3HeB/FeJ, a high LPS responder strain, did not result in any detectable MLC activity indicating that the defect was not associated with the M locus. A backcross linkage analysis using (C3H/HeJ X CWB) F_1 mice X C3H/HeJ mice revealed that the low LPS response may be due to a single gene which was not linked to H-2 expression, was not linked to heavy Ig chain allotype, and was not sex linked. Experiments by Rosenstreich and Glode (52), using C3H/HeJ and C3H/HeN (a high LPS responder strain), also showed that the defect was not associated with the H-2 locus. Skidmore et al (59) also showed that the defect which limited the immune response to LPS also limited the mitogenic and polyclonal response to LPS as well as preventing LPS from interfering with the induction of tolerance.

To determine if the defect in C3H/HeJ mice to respond

normally to LPS was located on B cells or whether the defect was associated with other cell types that regulated the expression of B cells, *in vivo* and *in vitro* cell mixing experiments were performed. Adult thymectomized-lethally irradiated C3H/HeJ mice reconstituted with high LPS responder C3HeB/FeJ bone marrow cells supported immune and mitogenic responses to LPS (71). Further, adult thymectomized-lethally irradiated C3H/HeJ mice reconstituted with both C3HeB/FeJ bone marrow and C3H/HeJ thymus cells supported LPS responses indicating no suppressor activity by the C3H/HeJ thymus cells (71). When C3H/HeJ animals were reconstituted with C3H/HeJ bone marrow cells and C3HeB/FeJ thymus cells, there was a failure to respond to LPS. This indicated that the C3H/HeJ low LPS response could not be attributed to a lack of some T cell function (71). Watson and Riblet (71) and Skidmore *et al* (59) performed *in vitro* cell mixing experiments using either C3HeB/FeJ or C3H/St (both high LPS responder strains) cells with C3H/HeJ cells. No MLC reactions were observed nor did the C3H/HeJ cells suppress or enhance the mitogenic response of C3HeB/FeJ or C3H/St cells. Reciprocal cell mixing experiments with purified

T cells or macrophages did not result in enhancement or suppression of the mitogenic response to LPS by the high LPS responder cells.

A recent report by Glode et al (21) has demonstrated that C3H/HeJ mice have no significant difference from high responder strains in the percentage of B cells or the total number of spleen cells. These studies also indicated that the macrophage did not play a suppressive or enhancing role.

Radiolabeled LPS was found to bind equally well to C3H/HeJ or C3HeB/FeJ spleen cells (71) which indicated that the genetic defect limiting the C3H/HeJ LPS response did not quantitatively reduce the binding of LPS to the B cells.

Statement of Thesis

The observations which indicated that the C3H/HeJ mouse is a low responder to LPS led to research which demonstrated that the low response was due to a genetic defect in the C3H/HeJ B cells. This defect has been proposed to be the inability of the B cells to enter an inductive pathway for blastogenesis after binding the LPS.

This central failure to enter blastogenesis has been the basis for explaining: 1) the low or absent mitogenic response to LPS by C3H/HeJ cells, 2) lack of T-dependent adjuvant activity by LPS, 3) failure to inhibit the induction of tolerance, 4) the very small polyclonal response, and 5) the anomalous antibody response. These observations on the C3H/HeJ strain have also served to amplify the proposal that the processes of antibody induction and synthesis are linked and that a mitogenic signal is sufficient by itself to induce B cell activation to antibody producing cells.

However, there is a paucity of data pertaining to the antibody response by this mouse strain and this aspect was examined. The demonstration of anti-LPS responses to LPS, E. coli bacterin, or NPP would not detract or contradict previous models of immune induction based on investigations with the C3H/HeJ mouse. Rather, positive responses could indicate that the specific response is dependent upon the endotoxin preparation used. In this respect I have concentrated on one particular LPS preparation and the research was designed to examine the following points:

1) Does the C3H/HeJ animal yield primary and

secondary antibody responses, both *in vivo* and *in vitro*, to the thymus-dependent antigen sheep erythrocytes?

2) Does the C3H/HeJ mouse give primary and secondary antibody responses, both *in vivo* and *in vitro*, to the thymus-independent antigen LPS?

3) Do the C3H/HeJ mice make primary and secondary responses *in vivo* and *in vitro* to LPS when the LPS is presented on bacterin rather than as a purified antigen extracted from the bacterin?

4) Do C3H/HeJ animals respond to the nonmitogenic O-polysaccharide of LPS as supplied by NPP, and can NPP prime these animals for a secondary response to LPS?

5) Is the *E. coli* 0113 bacterin mitogenic for C3H/HeJ spleen cells?

6) Do C3H/HeJ mice respond normally to other thymus-independent antigens such as the Vi antigen, Vi-positive *Citrobacter freundii* bacterin, and polyvinylpyrrolidone?

METHODS AND MATERIALS

Animals

Balb/c, C3H/HeJ, and F_1 (Balb/c X C3H/HeJ) mice were used in this investigation. The Balb/c animals were from stock maintained in our laboratory. The C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). F_1 mice were derived by crossing female C3H/HeJ with male Balb/c mice. The animals were maintained on acidified, chlorinated water and sterilized Purina Lab Chow 5010 *ad libitum*. All experiments were performed on age-matched mice of both sexes two to four months of age.

Antigens and In Vivo Immunizations

Sheep Erythrocyte Antigen

Sheep erythrocytes (SRBC) from different animals vary in the capacity to successfully immunize *in vitro* culture systems. Therefore, twenty-six blood samples, in Alsever's solution, (Colorado Serum Co., Denver, CO) were individually tested. Sheep No. 12 was selected as the best donor because erythrocytes from this animal elicited the highest responses. For primary and secondary *in vivo* immunizations, SRBC from sheep No. 12 were washed twice in sterile phosphate buffered saline (PBS) and resuspended to 20% in PBS. Mice received 0.1 ml of the SRBC suspension

intravenously (i.v.) via the lateral tail vein.

Lipopolysaccharide Antigen

Lipopolysaccharide, extracted from E. coli 0113 by the phenol-water method of Westphal (74), was kindly supplied by Dr. J. A. Rudbach (Univ. of Montana, Missoula, MT). Mice were immunized for primary responses by injecting i.v. 1μg of LPS in 0.1 ml PBS. Secondary responses were elicited by priming the animals with 1μg LPS in 0.1 ml PBS i.v. followed either 9 or 14 days later with a boosting dose of 1μg LPS in 0.1 ml PBS i.v. All LPS preparations were filter sterilized by passage through a 0.22μ millipore filter using a Swinnex filter assembly and syringe.

Native Protoplasmic Polysaccharide

Native protoplasmic polysaccharide was extracted from the protoplasmic fraction of E. coli 0113 with cold trichloroacetic acid, as described by Anacker et al (2). The NPP was prepared and kindly supplied by Dr. J. A. Rudbach. The immunization schedule for primary and secondary responses was the same as for the LPS immunizations (day 9). All NPP preparations were filter sterilized.

Escherichia coli 0113 Bacterin

A stock bacterin containing approximately 3 X 10^{10} heat killed E. coli 0113 cells per ml was a gift from Dr. J. A. Rudbach. Whole cells, possessing LPS, were used as an antigen in both in vivo and in vitro studies. The bacterin was washed four times in PBS and resuspended in PBS to a concentration of 1 X 10^{10} cells per ml. Mice received 1 X 10^{9} cells in 0.1 ml PBS i.v. for both primary and secondary injections. For in vitro secondary responses, mice were first primed with 1 X 10^{9} cells in 0.1 ml PBS i.v., and nine days later sacrificed for culturing.

Vi Antigen

Vi antigen, extracted from Citrobacter freundii and purified by an electrophoresis procedure (29), was kindly donated by Dr. F. G. Jarvis (Idaho State Univ., Pocatello, ID). Mice were injected i.v. with 0.2 ml of a saline solution of Vi, the concentrations varying as indicated in the "Results" section.

Citrobacter freundii Bacterin

From a culture stock donated by Dr. F. G. Jarvis, a stock bacterin containing approximately 4.4 X 10^{9} C. freundii Vi-positive cells per ml in 0.3% formalized saline

(9) was prepared by Dr. N. D. Reed (Montana State Univ., Bozeman, MT). The bacterin was washed four times in PBS and resuspended in PBS. Mice received 0.2 ml of the cell suspension i.v. at concentrations indicated in the "Results" section.

Polyvinylpyrrolidone

Polyvinylpyrrolidone, type K-90, molecular weight 3.6×10^4 daltons, (GAF Corp., New York, NY) was dissolved in PBS and injected i.v. at a concentration of 0.25μg in 0.25 ml per mouse.

Cell Cultures for Generation of In Vitro Plaque Forming Cells

Cell Cultures

Spleen cell cultures were prepared using a modification by Click et al (13) of the original in vitro technique described by Mishell and Dutton (42). Briefly, the procedure was as follows. Mice were killed by cervical dislocation. The spleens were aseptically removed and transferred to a sterile 60 X 15 mm plastic Petri dish (Falcon Plastics, No. 3002) containing 5 ml of cold sterile Hank's balanced salt solution (HBSS, Microbiological Associates, Los Angeles, CA, No. 10-518). A maximum of two

spleens per dish was allowed. Single cell suspensions were prepared by extruding the splenic contents from the spleen capsule and dissociating the mass by repeated aspirations through a 1 ml sterile plastic pipet (Falcon Plastics, No. 7521). This was followed by sedimentation of the entire suspension in sterile plastic test tubes (Falcon Plastics, No. 2054) to exclude the tissue fragments and debris from the cells. The resulting single cell suspension was centrifuged at 300 X g for 7 minutes and the cells resuspended in complete culture medium. Cultures were established in sterile 35 X 10 mm plastic Petri dishes (Falcon Plastics, No. 3001). The cell concentration was adjusted to 7 X 10^6 cells per ml and 2 ml of the cell suspension added to each dish. All cultures were maintained (unless otherwise indicated) for 5 days in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 within lucite chambers at 37°C on a continuous rocking platform. No daily feedings were required, and rocking (7-10 cycles per minute) was terminated after 3.5 days.

Culture Medium

The following culture medium was employed. (Reagents were purchased from Microbiological Associates,

Los Angeles, CA, except where indicated.)

Part One:

	Conc.	Stock #	Amount
Sterile deionized water		17-724	71.3 ml
Hank's BSS	10X	10-518	10.0 ml
Essential amino acids	50X	13-606	4.0 ml
Nonessential amino acids	100X	13-114	5.0 ml
Nucleic acid precursors	1X, stock	***	2.5 ml
Essential vitamins	50X	13-607	2.0 ml
Sodium pyruvate	100mM	13-115	2.5 ml
L-glutamine	200mM	17-605	2.0 ml
Penicillin-Streptomycin	5000 units per ml	17-603	1.0 ml

*** The stock solution of nucleic acid precursors was prepared by dissolving 50 mg each of adenosine, guanosine, uridine, and cytosine (precursors purchased from Grand Island Biological Co., Grand Island, NY., Adenosine, #60030; quanosine, #60305; uridine, #60570, cytosine, #60130) in 50 ml of sterile deionized water. To this solution was added 0.2ml of 2M NaOH to prevent precipitation of the reagents. The stock was filter sterilized

by passage through a Swinnex millipore assembly using a 0.22μ filter.

Part Two:

The medium described in Part One was adjusted to a pH of 7.0 with approximately 0.47ml of 2M NaOH and designated "incomplete medium".

Part Three:

Final preparation of the medium consisted of mixing with 44.5ml incomplete medium the following reagents:

2-mercaptoethanol**	***	0.05 ml
Fetal calf serum	14-414	5.0 ml
Sodium bicarbonate	17-613	0.48 ml

** The stock solution of 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, #M-6250) was prepared by adding 0.35 ml of 2-ME into 100 ml of Hank's balanced salt solution.

In Vitro Immunizations

In vitro immunizations were performed with the following antigens: SRBC, LPS, and E. coli bacterin.

Sheep Erythrocytes

SRBC from sheep No. 12 were washed three times in

sterile PBS and resuspended to 1.5% in complete culture medium. Successful immunization was achieved by adding 0.05 ml of the SRBC suspension to each culture.

Lipopolysaccharide

Filter sterilized LPS solutions were added to each culture in 0.05 ml volumes. The concentrations ranged from 1 to 50μg LPS per culture.

Escherichia coli Bacterin

E. coli 0113 cells were washed four times in sterile PBS and resuspended in complete culture medium. All immunizing doses were given in 0.05 ml volumes and the concentrations of bacterin adjusted to cover the range of 5×10^6 to 1×10^8 cells per culture.

Localized Hemolysis-in-Gel Assay

The plaque forming cell (PFC) response of in vivo or in vitro experiments was assayed by a slide modification (42) of the hemolysis-in-gel technique of Jerne (31). Agarose (Sigma Chemical Co., St. Louis, MO) or Seaplaque agarose (Microbiological Associates, Bethesda, MD) was used at concentrations of 0.6% and 0.9% respectively in Dutton's balanced salt solution (42).

Preparation of Cells for Plaque Assay

Spleen Cells, In Vivo Donors

Spleens were aseptically removed and a single cell suspension in PBS was prepared as described in the "Cell Cultures for Generation of *In Vitro* PFC" section. The final suspension of the cells in 2.5 ml Dutton's balanced salt solution per spleen was done in sterile plastic test tubes.

Spleen Cells, In Vitro Cultures

Each *in vitro* experiment employed triplicate sets of cultures which were combined upon harvesting. A tygon policeman was used to scrape and remove the cells from the bottoms of the culture dishes. The cell suspension was transferred with a Pasteur pipet into plastic test tubes and centrifuged for 5 minutes at 300 X g. The cell pellet was resuspended in 1 ml Dutton's balanced salt solution and used as the "undiluted" sample in the plaque assay.

Antigen Coating of Sheep Erythrocytes

LPS-SRBC

To determine specific PFC to LPS, NPP, or *E. coli* 0113 bacterin, SRBC were coated with LPS from *E. coli* 0113

by the method of Neter et al (46). 1 mg LPS per ml PBS was boiled for 2.5 hours. Coating of SRBC was achieved by incubating 1 mg boiled LPS diluted 1:10 in PBS per 0.3 ml of packed SRBC for 0.5 hours at 37°C. The SRBC were then washed three times in PBS, and a 10% suspension of LPS-coated-SRBC was used for plaquing. Assays were performed on animals or in vitro cultures on the days indicated in the "Results" section.

Vi-SRBC and Plaquing Procedure

The plaquing procedure for the Vi antigen was developed by Reed et al (49) to detect Vi specific PFC from purified Vi or C. freundii bacterin immunizations. Coating the SRBC with Vi antigen was accomplished by mixing equal volumes of a 10% SRBC suspension in PBS with a 0.05μg Vi per ml PBS solution and incubating at 37°C for 2 hours. The SRBC were then washed four times in 10 volumes of PBS and resuspended in PBS at a concentration of 8% for the PFC assay. After an initial 2 hour incubation at 37°C in a humid atmosphere, the slides were flooded with a 1:10 dilution in Dutton's balanced salt solution of guinea pig complement. An optimal dilution of rabbit anti-mouse gamma-globulin (1:400) was used to

facilitate plaque formation; the facilitating antiserum was added to the 1:10 complement. An additional 1 hour incubation at 37°C was allowed for the plaques to develop. Assays were performed on day 5 following immunization.

PVP-SRBC and Plaque Procedure

The number of PFC to PVP was determined 5 days after immunization by using SRBC coated with PVP as described by Rotter and Trainin (53). SRBC were washed three times in PBS and resuspended to 5% in PBS. Equal volumes of cells and a tannic acid solution (0.1 mg tannic acid per ml PBS) were incubated at room temperature for 15 minutes; the tannic acid solution had been made 10-12 hours previously. The SRBC were washed three times in PBS and divided into two aliquots. One-half of the cells were resuspended to 8% in Dutton's balanced salt solution and served as the tannic acid treatment control in the plaque assay. The rest of the cells were resuspended to 5% in PBS, and equal volumes of the SRBC suspension and a PVP solution (0.1 mg PVP per ml PBS) were incubated for 15 minutes at room temperature. The cells were washed three times and resuspended to 8% in Dutton's balanced salt solution for plaquing.

Stimulation of Cell Cultures by Mitogens

Mitogens

Spleen or thymus cells were examined for a mitogenic response to four mitogens: Phytohemagglutinin (PHA) (Difco Labs, Inc., Detroit, MI, No. 3110-56); Concanavalin A (Con A) (Miles Labs, Inc., Kankakee, IL); LPS and E. coli 0113 bacterin. The PHA, Con A, and LPS mitogen preparations were made in PBS, filtered sterilized, and stored at -20°C. The E. coli bacterin was washed four times in sterile PBS and resuspended in sterile PBS. The concentrations of the mitogens are indicated in the "Results" section.

Cell Cultures

Cultures for mitogen studies were prepared from both spleen and thymus cells. Cell suspensions of the organs were made exactly as previously described in "Cell Cultures for Generation of In Vitro PFC", through the sedimentation step. The spleen or thymus cell suspension was then centrifuged for 5 minutes at 300 X g and resuspended in 5 ml (per two spleens or 5 thymuses) of cold sterile tris-NH_4Cl (see below for formulation) for 5 minutes on ice to eliminate the mouse erythrocytes. The cells were washed

twice in cold PBS supplemented with 2% fetal calf serum, centrifuged for 5 minutes at 300 X g, and resuspended in complete mitogen medium (see below for formulation). The cell concentration was adjusted to 5 X 10^6 viable cells per ml as determined by the trypan blue dye exclusion procedure.

Triplicate cultures were established in sterile Microtest II tissue culture plates (Falcon Plastics, #3040) using 0.2 ml of the cell preparation per well. The cultures were incubated at 37°C in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 for 48 hours before pulsing with tritiated thymidine.

Formulations

Tris-NH_4Cl

a) Dissolve 1.66g NH_4Cl in 200 ml distilled H_2O.

b) Dissolve 0.51g Tris in 25 ml distilled H_2O. Adjust pH to 7.65 with HCl.

c) Mix 180 ml "a" with 20 ml "b". pH to 7.2 with HCl.

d) Filter sterilize the solution and store in 250 ml sterile plastic flask. (Falcon, #3024)

Complete Mitogen Medium

	Stock #	Amt.
RPMI 1640	12-702	93 ml
L-glutamine	17-605	1 ml
Penicillin-streptomycin	17-603	1 ml
Fetal calf serum	14-414	5 ml

(All reagents purchased from Microbiological Associates, Los Angeles, CA)

Pulsing Cultures

Forty-eight hour old cultures were pulsed with thymidine-methyl-^{3}H (New England Nuclear, Boston, MA, No. NET-027X) by adding 1μCi delivered in 25λ of RPMI 1640 to each well. (The NET-027X preparation had an activity of 0.25 mCi per 0.5 ml. Therefore, 0.2 ml of isotope into 2.3 ml RPMI was equal to 40μ Ci per ml or 1μCi per 25λ).

Harvesting Cultures and Liquid Scintillation Assays

The Microtest II plates were centrifuged at 350 X g for 8 minutes to insure adherence of the cells to the bottom of the wells. The plates were "flicked" to remove the supernatant from all the wells simultaneously. The following trichloroacetic acid (TCA) procedure was

performed three times to precipitate the DNA. Each well of the Microtest plate was filled using a Pasteur pipet with 7 drops of 5% aqueous TCA, directly centrifuged at 350 X g for 7 minutes and then "flicked". Following the final TCA treatment, 5 drops from a Pasteur pipet of 1 M NaOH was added to each well and allowed to dissolve the precipitate for 2 hours.

Separate Pasteur pipets were used to transfer the contents of each well to liquid scintillation vials (Beckman Instruments, Inc., Salt Lake City, UT; "Extra Vials", No. 161698) containing 0.8 ml of 0.5 M acetic acid. To each vial was then added 10 ml of Aquasol, an universal liquid scintillation counting cocktail (New England Nuclear, Boston, MA; No. NEF-934) and shaken to insure mixing. All vials were counted in a Beckman LS-100C liquid scintillation system.

RESULTS

In Vivo Response of Balb/c, C3H/HeJ, and F_1 Mice to SRBC, Vi, PVP, NPP, LPS, and Bacterins

A series of in vivo studies was performed to compare the immune response of Balb/c, C3H/HeJ, and F_1 mice to several antigens. Tables 1 through 6 pertain to the in vivo work and all individual results may be found in the "Appendix to Results" section.

Primary and Secondary Responses by Mice to SRBC

The immune response to the thymus-dependent antigen SRBC was examined in Balb/c, C3H/HeJ, and F_1 mice (Table 1). Sultzer (65) reported that C3H/HeJ mice made normal primary responses to SRBC. My data indicate that the primary PFC responses of the three strains are of similar magnitude. I have further demonstrated that the C3H/HeJ mice make a normal secondary response to SRBC, approximately ten times the primary response as assayed by facilitating serum to develop the IgG plaques. (F_1 animals were not tested).

Response by Mice to Vi Antigen

The Vi antigen, a polymer of N-acetyl-D-galactosaminuronic acid and produced by some members of the

Enterobacteriaceae, has been demonstrated by Reed et al (50) to be a T cell independent antigen in that thymus-derived cells were not required for an immune response. Shown in Table 2 are the PFC responses of Balb/c and C3H/HeJ mice to Vi and Vi-positive Citrobacter freundii cells. Mice were injected with antigen on day 0 and assayed on day 5. Although the C3H/HeJ mice responded at approximately one-fourth the level of the Balb/c mice, my experiments establish that both strains do make primary responses to Vi or Vi-positive bacterin. Facilitating serum was used to increase the size and clarify the plaques for counting; however these facilitated plaques were shown by Reed et al (49) to be IgM plaques.

Response by Mice to PVP

The antigen PVP is considered to be a T-independent antigen based on experiments demonstrating that: 1) neonatal thymectomy did not alter the antibody response in adult mice (5), 2) adult thymectomized, lethally irradiated mice reconstituted with syngeneic bone marrow cells responded to PVP (6), 3) congenitally thymusless nude mice responded to PVP (34). Thymus cells, however, have been demonstrated to exert a regulatory function over the PVP

response (34,53). I have shown (Table 3) that Balb/c, C3H/HeJ, and F_1 mice immunized i.v. with 0.25µg PVP on day 0 and assayed on day 5 are capable of mounting nearly identical primary PFC responses to this antigen.

Responses by Mice to LPS

The original observation that C3H/HeJ mice are low primary (IgM) responders to the thymus-independent antigen LPS (5,39,51) was made by Watson and Riblet (66). The primary responses (Table 4) by C3H/HeJ mice to LPS confirm these initial observations and are in agreement with the results reported by Skidmore et al (59). C3H/HeJ animals make very small primary responses to LPS compared to Balb/c or F_1 mice.

Because secondary responses to LPS by C3H/HeJ mice have never been previously reported, these experiments were performed using two time schedules. In experiment I, animals were primed with LPS on day 0, boosted on day 14, and assayed on day 18. The results indicate that the residual response (R^o = that response remaining 18 days after the priming injection) by Balb/c and C3H/HeJ mice to the priming LPS dose is similar and very low. The boosting injection, four days before assaying, results in a

secondary response by the Balb/c mice and no secondary response by the C3H/HeJ mice. In experiment II, mice were primed with LPS on day 0, boosted on day 9, and assayed on day 13. The residual responses by the Balb/c and F_1 mice are similar and about three times greater than the C3H/HeJ residual response. While there is a proportionate three fold increase in the secondary responses over the primary responses to LPS in all three strains, I do not consider the C3H/HeJ response of 1500 PFC's as being really indicative of a secondary response because the number of animals in the test group was small and because the C3H/HeJ response to a second injection of LPS is clearly more than an order of magnitude less than the number of PFC's generated by the Balb/c or F_1 mice. These results confirm that C3H/HeJ mice are low primary responders to LPS and indicate that C3H/HeJ mice do not give secondary responses to LPS when immunized on either time schedule.

Primary and Secondary Responses by Mice to E. coli Bacterin

Experiments with bacterin were performed for two reasons: Because the C3H/HeJ mice respond at a very low level to an LPS preparation, it was of interest to determine

how they would respond to LPS presented on E. coli 0113 bacterin. Secondly, when in vitro experiments are performed, soluble antigens are much less effective in eliciting a response than are particulate antigens. Thus, I wanted also to establish an in vivo correlate for the in vitro work with bacterin. Presented in Table 5 are the results of two experiments using bacterin. The primary PFC response by C3H/HeJ mice to E. coli 0113 cells appeared normal compared to the response by Balb/c mice. This indicates that antibody to LPS can be made. A secondary challenge with bacterin yielded somewhat variable results. The C3H/HeJ mice appeared in experiment I to give a second response equal to the primary, while the results of experiment II indicate that a secondary response only about twice the magnitude of the primary could be elicited. I interpret these data as indicating no differences between the primary responses to bacterin by Balb/c and C3H/HeJ mice, and that C3H/HeJ mice do not make secondary anti LPS responses to the E. coli bacterin but rather second primary responses that may be marginally greater than the first primary. Certainly the C3H/HeJ mice do not demonstrate the 10 fold increase in PFC's as do the

Balb/c mice to a second challenge with bacterin.

Response by Mice to NPP, LPS, and an NPP Priming-LPS Challenge

NPP extracted from E. coli was determimed by Rudbach et al (55) to be a polysaccharide with an average molecular weight of 168,000 daltons. Immunochemically, NPP does not contain lipid A, 2-keto-3-deoxyoctulosonate, or heptose but does contain the O-antigenic determinants that cross-react completely with the O-antigenic determinants of homologous LPS (67). Von Eschen and Rudbach have demonstrated that although NPP was not mitogenic for B cells, NPP could elicit a primary response with specificity toward the O-specific antigens of NPP or homologous LPS (67). Von Eschen and Rudbach have further shown that mice did not make a secondary response to NPP, and established that NPP could prime mice for a secondary response when subsequently challenged with LPS (67).

Experiments were performed to determine whether or not the C3H/HeJ mice could be primed with NPP to give a secondary response to LPS. The results given in Table 6 show how Balb/c and C3H/HeJ mice responded to priming and secondary injections of NPP or LPS. Also shown are the

responses to a secondary challenge with LPS when first primed with NPP. Balb/c and C3H/HeJ mice gave a similar primary response to 1μg NPP (10μg data not shown, but these primary responses to NPP were also similar), and LPS elicited a low primary response in the C3H/HeJ compared to the Balb/c mice.

For determining secondary responses to NPP or LPS, 1μg of each antigen was used for priming and secondary challenge. No secondary response by C3H/HeJ mice to NPP was found. While the Balb/c mice showed a slightly greater second NPP response over the primary, this is not considered as significant. The Balb/c animals made an uncommonly high secondary response to LPS. The C3H/HeJ mice were low responders to a second challenge with LPS as expected; the 763 PFC's observed is less than two times the primary response and is considered as indicative of only a second primary. Priming by NPP is shown in the last entry of Table 6. It is evident that NPP did prime the Balb/c mice to give a very strong secondary response to LPS. The NPP does not appear to have primed the C3H/HeJ animals for a secondary LPS response.

My *in vivo* work presented in C3H/HeJ mice confirms that they are: 1) normal primary responders to the thymus-dependent antigen SRBC, 2) low primary responders to the T-independent antigen LPS. New information on the C3H/HeJ strain includes: 1) that C3H/HeJ mice can make normal secondary IgG responses to SRBC, 2) that C3H/HeJ mice are able to give primary responses to the T-independent antigens Vi and Vi-positive bacterin, 3) that C3H/HeJ mice make normal primary responses to the T-independent antigen PVP, 4) C3H/HeJ mice do not make secondary responses to LPS, 5) C3H/HeJ mice make a normal primary response to *E. coli* 0113 bacterin, but do not make secondary responses to the bacterin, 6) C3H/HeJ animals make a normal primary response to NPP, but NPP does not appear to prime these mice for a secondary response to an LPS challenge.

TABLE I

In Vivo Primary and Secondary Responses of Mice to SRBC

	PFC/Spleen[a]					
	Balb/c		C3H/HeJ		F_1	
	Dir.[b]	Fac.	Dir.	Fac.	Dir.	Fac.
1^{o}[c]	63,594	57,656	88,281	83,750	63,600	59,760
R^{o}	2,100	9,313	825	11,613		
2^{o}	88,214	1,418,750	32,656	895,313		

a) 1^{o} and 2^{o} doses were 0.2 ml of 10% SRBC in PBS given i.v. R^{o} and 2^{o} mice were injected on day 0. 1^{o} and 2^{o} mice were injected on day 20. Assay was performed on day 24.

b) Dir. = direct plaques. Fac. = facilitated plaques: Rabbit anti-mouse globulin added to complement to develop IgG plaques.

c) 1^{o} = primary response. R^{o} = the residual response seen on the day of the assay to a priming dose given on day 0. 2^{o} = secondary response.

TABLE II

In Vivo Response of Mice to Vi and Vi-Positive *C. freundii* Cells

	PFC/Spleen	
Dose[a]	Balb/c	C3H/HeJ
1μg Vi	26,900	5,750
100μg Vi	1,875	50
1 X 10^9 cells	58,163	16,250

a) All mice were injected i.v. at the doses indicated in 0.2 ml PBS. The plaques were facilitated with rabbit-anti-mouse globulin serum.

TABLE III

In Vivo Response of Mice to PVP

PFC/Spleen[a]		
Balb/c	C3H/HeJ	F_1
12,675	13,080	12,030

a) All mice were injected i.v. with 0.25μg of PVP in 0.25 ml of PBS.

TABLE IV

In Vivo Primary and Secondary Responses of Mice to LPS

		PFC/Spleen[a]		
		Balb/c	C3H/HeJ	F_1
Exp.[b] I	1^o	3,740	430	
	R^o	83	100	
	2^o	22,475	290	
Exp.[c] II	1^o	6,163	538	6,470
	R^o	1,150	325	1,050
	2^o	21,100	1,562	17,038

a) Primary and secondary doses of LPS were 1μg in 0.1 ml PBS given i.v.

b) R^o and 2^o mice were injected on day 0. 1^o and 2^o mice were injected on day 14. The assay was performed on day 18. Also see footnote c, Table 1.

c) R^o and 2^o mice injected on day 0. 1^o and 2^o mice injected on day 9. Assay done on day 13. Also see footnote c, Table 1.

TABLE V

In Vivo Primary and Secondary Responses of Mice to *E. coli* Cells

		PFC/Spleen[a]	
		Balb/c	C3H/HeJ
Exp. I[b]	1^o	40,013	25,675
	R^o	1,475	1,325
	2^o	310,938	25,163
Exp. II[b]	1^o	20,500	33,500
	R^o	1,300	1,340
	2^o	275,630	58,250

a) All 1^o, R^o, and 2^o doses were 1×10^9 cells of bacterin given i.v. in 0.1 ml of PBS.

b) R^o and 2^o mice were injected on day 0. 1^o and 2^o mice were injected on day 9. The assay was performed on day 13. Also see footnote c, Table 1.

TABLE VI

In Vivo Primary and Secondary Responses of Mice to NPP, LPS, and the Secondary Response to LPS by Mice First Primed With NPP

	PFC/Spleen	
	Balb/c	C3H/HeJ
NPP 1°[a]	1,410	1,200
LPS 1°	6,600	390
NPP 2°[b]	3,094	1,157
LPS 2°	116,094	763
NPP primed-LPS 2°[c]	69,063	525

a) NPP and LPS 1° doses were 1μg in 0.1 ml PBS given i.v. on day 0. Assay was done on day 4. The primary response experiment was done several days after the secondary and NPP-priming experiment.

b) NPP and LPS priming doses were 1μg in 0.1 ml PBS given i.v. on day 0. The 2° injections of NPP and LPS were 1μg in 0.1 ml PBS given i.v. on day 9. Assays were done on day 13.

c) The NPP priming dose was 1μg in 0.1 ml PBS given i.v. on day 0. LPS was given on day 9, 1μg in 0.1 ml PBS, i.v. Assay was performed on day 13.

In Vitro Response of Balb/c, C3H/HeJ, and F_1 Mice to SRBC, LPS, and *E. coli* Bacterin

A series of *in vitro* studies was performed to compare the *in vitro* with the *in vivo* PFC immune response of C3H/HeJ mice with Balb/c and F_1 mice. The *in vitro* generation of PFC responses in C3H/HeJ mice to SRBC, LPS, or *E. coli* bacterin has not been reported previously. The results in Tables 7-9 are expressed as PFC per culture. All primary and secondary cultures were made by pooling spleens from 3-4 mice, and all residual cultures were made from the spleens of 1-2 mice per experiment.

Primary and Secondary Responses by Balb/c, C3H/HeJ, and F_1 Cell Cultures to SRBC

The results of two experiments using the antigen SRBC are presented in Table 7. I have shown that the C3H/HeJ and F_1 animals are capable of giving a primary response to SRBC; these animals were routinely found to respond at a somewhat lower level than the Balb/c mice *in vitro*. Secondary responses *in vitro* were performed by immunizing the mice with SRBC and fifteen days later the animals were sacrificed and the spleen cells cultured with SRBC. Thus,

the primary responses are with unprimed mice and immunized *in vitro*, while the secondary responses are from mice primed *in vivo* and immunized *in vitro*. Residual responses are derived from mice primed *in vivo* and their spleen cells subsequently cultured without antigen. No practical primary and secondary *in vitro* immunization could be performed with the time schedule used because the cell cultures rapidly deteriorate after about five days. In both experiments I have demonstrated that C3H/HeJ mice do give secondary responses to SRBC as evidenced by both the small residual response compared to the secondary response, and the switch from IgM to IgG antibody as determined by the requirement for facilitating serum to develop the heightened number of plaques. Primary responses were not assayed for IgG plaques using facilitating serum because it was assumed that the primary response would be IgM antibodies.

In Vitro Primary and Secondary Responses of Cell Cultures to LPS

The generation of an *in vitro* PFC response to soluble antigens is generally very difficult to demonstrate. Numerous attempts with Vi and PVP resulted in never more

than 200 PFC per culture and averaged around 50-75 (data not shown). My efforts were concentrated on LPS. All *in vitro* LPS experiments employed LPS at a concentration of 20μg per culture. This dose was used because my earlier work showed no discernable difference in PFC responses using 10-100μg LPS per culture. There was only a small decrease in the PFC response from 0.01 to 10μg LPS per culture.

The primary *in vitro* response to LPS, given in Table 8, represents an average response from five experiments with the Balb/c mice and two experiments with the C3H/HeJ and F_1 mice. The primary responses were all quite low but similar. Secondary responses are also low. Because of the difficulty in eliciting *in vitro* responses to soluble antigens, my interpretation of the data is conservative. Whether or not 20 to 50 PFC's represent a primary response to LPS is not certain because this low value may either reflect a specific anti-LPS response or it may reflect low avidity antibody elicited as a polyclonal response by the mitogens present in the fetal calf serum. That is, assaying the specific response of a cell culture immunized with LPS by plaquing the cultured cells with

SRBC and with SRBC-LPS, and deriving the specific anti-LPS response by subtracting the background (anti-SRBC) plaques from the number of anti-SRBC-LPS plaques may not be justified. The problem arises that perhaps the observed anti-LPS response was a consequence of merely providing more antibody binding sites (SRBC-LPS) that fortuitously bound low avidity antibody generated from the nonspecific polyclonal response. Thus I do not conclude that the C3H/HeJ spleen cells gave primary responses to LPS *in vitro*. The secondary responses to LPS are also analyzed in a conservative manner. The Balb/c and F_1 spleen cell cultures may have given a secondary *in vitro* anti-LPS response while the C3H/HeJ cells probably did not. Again, I will point out that the secondary responses are derived from animals which were primed *in vivo* and the subsequent cell cultures boosted with antigen *in vitro*.

In Vitro Primary and Secondary Responses by Cell Cultures to E. coli Bacterin

I performed two experiments using *E. coli* 0113 bacterin to immunize *in vitro* cultures. These experiments were done to determine how the C3H/HeJ mice would respond

when LPS was presented in a particulate form rather than as a purified antigen. The data in Table 9 indicate that the Balb/c and C3H/HeJ spleen cells might make low primary responses to the bacterin. The magnitude of the Balb/c and C3H/HeJ primary response to E. coli bacterin was very similar to the responses elicited by Vi-positive C. freundii bacterin (data not shown). When the mice were primed in vivo and their spleen cells immunized in vitro with E. coli bacterin, a very strong secondary response was elicited. These results establish that the C3H/HeJ cells respond very well in vitro to a secondary challenge with LPS presented on bacterin.

I have established in these in vitro reports that: 1) C3H/HeJ spleen cells respond to give near normal in vitro primary and secondary PFC responses to SRBC. Although the response is lower than that seen in Balb/c spleen cell cultures, this is probably in accordance with the observations by many investigators that various strains respond at different levels to SRBC in vitro, 2) C3H/HeJ spleen cells do not appear to make primary in vitro responses to LPS, and do not appear capable of giving secondary in vitro responses to LPS, 3) the C3H/HeJ strain may

perhaps make a primary *in vitro* response to *E. coli* 0113 bacterin which possesses LPS. Most importantly, the C3H/HeJ spleen cells respond very well to a secondary *in vitro* immunization with *E. coli* bacterin. These results can be interpreted to indicate that the C3H/HeJ mouse is a low responder to purified LPS 0113, but is a high responder to LPS presented on *E. coli* 0113 bacterin.

TABLE VII

Primary and Secondary Responses

by Cell Cultures to SRBC

		PFC/Culture					
		Balb/c		C3H/HeJ		F_1	
		Dir.	Fac.	Dir.	Fac.	Dir.	Fac.
Exp.[a] I	1^o	4,730	---	437	---	827	---
	R^o	233	827	50	250	117	343
	2^o	1,500	33,960	400	21,960	433	24,960
Exp.[a] II	1^o	1,293	---	477	---		
	R^o	483	1,083	40	283		
	2^o	1,117	100,000	477	41,667		

a) Primary responses are primary *in vitro* responses immunized as per Materials and Methods section. R^o (residual response, see footnote c, Table 1) responses are from mice immunized *in vivo* with 0.1 ml of 20% SE suspension in PBS, i.p. on day 0. The spleens were removed on day 15 and cultured for 5 days without any *in vitro* immunization with SE. 2^o responses are from mice immunized *in vivo* with 0.1 ml 20% SE suspension in PBS, i.p., day 0. The spleens were removed on day 15 and the cells cultured for 5 days with SE for a secondary *in vitro* immunization. Assays done after 5 days in culture.

TABLE VIII

Primary and Secondary Responses of Cell Cultures to LPS

	PFC/Culture		
	Balb/c	C3H/HeJ	F_1
1^{o}[a]	50	20	35
R^{o}[b]	93	10	77
2^{o}[b]	417	90	310

a) 1^{o} doses of LPS were 20μg/culture, or 10μg/ml.

b) R^{o} (see footnote c, Table 1) responses are from mice that were primed *in vivo* with 1μg LPS in 0.1 ml PBS, i.v., on day 0. The spleens were removed on day 8 and cultured for 5 days without any LPS. 2^{o} responses are from mice primed *in vivo* with 1μg LPS given in 0.1 ml PBS, i.v., day 0. The spleens were removed and the cells cultured for 5 days with LPS at a concentration of 20μg/culture. All assays were done on day 13.

TABLE IX

Primary and Secondary Responses of Cell Cultures to E. coli Bacterin

		PFC/Culture	
		Balb/c	C3H/HeJ
		100	90
Exp. I[a,b]	R^o	390	320
	2^o	9,540	9,740
	1^o	200	77
Exp. II[a,b]	R^o	717	334
	2^o	22,600	5,627

a) The optimum dose of bacterin was 1×10^7 cells/culture, or 5×10^6 cells/ml of culture.

b) R^o (see footnote c, Table 1) responses are from mice primed with 1×10^9 cells of bacterin in 0.1 ml PBS, i.v., day 0. The spleens were removed and the cells cultured on day 9 without any bacterin added to the cultures. 2^o responses are from mice which were primed with 1×10^9 cells of bacterin in 0.1 ml PBS, i.v., day 0. The spleens were removed and the cells cultured on day 9 with a secondary *in vitro* immunization of 1×10^7 bacterin cells/culture. Assays were done on day 14.

Mitogenic Responses by Balb/c, C3H/HeJ, and F_1 Mice Spleen and Thymus Cells to PHA, Con A, LPS, and *E. coli* Bacterin

Studies with mitogens were performed using spleen and thymus cells from Balb/c, C3H/HeJ, and F_1 mice. Each experiment used cells pooled from five animals per group. Two T-cell mitogens, PHA and Con A, were used for comparing the C3H/HeJ response to the responses by Balb/c and F_1 mice. These mitogens also served as a positive control on the mitogen assays. My primary concern was to compare the mitogenicity of the 0113 LPS preparation with the mitogenic activity of LPS-positive *E. coli* 0113 cells. One very plausable explanation for the low LPS responses by C3H/HeJ mice *in vivo* and *in vitro* might be related to alterations of the LPS induced during the phenol extraction. The bacterin might present the LPS in a form which allows a mitogenic response and subsequent antibody response. Numerous reports indicate that a mitogenic response is a necessary prerequisite step to antibody production. Thus, changes in the LPS induced during the extraction might have resulted in a structure which the C3H/HeJ B cells could not recognize. Past reports have

concluded that the C3H/HeJ strain is genetically unable to respond to endotoxin. My in vitro PFC results indicate that the strain does respond to endotoxin on bacterin.

Janossy and Greaves (27,28) have shown that PHA and Con A were mitogenic for T cells but not for B cells. Cortisone resistant T cells showed a greater mitogenic response than did unselected thymocytes to PHA and Con A; however, Con A could activate a considerable response in the latter population of cells.

The main mitogenic effect of LPS has been found to be exerted exclusively on B cells (3,11,20), and different experimental approaches have revealed that the lipid A moiety was responsible for the mitogenicity of LPS for B cells (4,11,47).

Mitogenic Response by Balb/c, C3H/HeJ, and F_1 Thymus Cells to PHA, Con A, LPS, and E. coli Bacterin

The data in Table 10 show the results of two mitogen studies on thymus cells from the three mice strains using PHA, Con A, LPS, and bacterin. Results at the optimum mitogen concentrations, as determined by the maximum cpm, are reported. (The PHA preparation yielded the optimum stimulation at 5µg per ml; it was tested over a range of

0.5, 2, 5, 10, and 25μg per ml. The Con A preparation gave the best stimulation at 2μg per ml; it was tested at 0.5, 1, 2, 5, and 10μg per ml. The LPS could be used over a wide range of concentrations from 10 to 50μg per ml with similar results. The 10μg per ml dose was selected. The bacterin was most mitogenic between 1 X 10^6 and 5 X 10^6 cells/ml; 1 X 10^6 cells/ml was selected. The bacterin was tested through a range of 1 X 10^5 to 1 X 10^8 cells/ml).

Both experiments on the thymus cells show the same trends. PHA stimulated the cells approximately 10 to 15 times over the background cpm. Con A was very mitogenic for all the thymus cells yielding about 100 to 500 times stimulation over background levels. LPS was mildly mitogenic for the Balb/c and F_1 thymus cells and not mitogenic for the C3H/HeJ thymus cells. Since LPS is a B cell mitogen, it is suggested that the observed stimulation may have resulted from a small proportion of B cells in the thymus cell preparation from recirculating peripheral B lymphocytes through the thymus. The observed stimulation might have also been due to the inadvertent inclusion of some of the small parathymic lymph nodes in

the thymus cell preparation. The E. coli bacterin was approximately as mitogenic for the thymus cells as was the LPS; however, the bacterin was also mitogenic for the C3H/HeJ thymus cells.

Mitogenic Responses by Balb/c, C3H/HeJ and F_1 Spleen Cells to PHA, Con A, LPS, and E. coli Bacterin

Mitogenic studies on the spleen cells from Balb/c, C3H/HeJ, and F_1 mice (Table 11) follow the same dose scheme which was employed for the thymus cells. The same spectrum of mitogen concentrations was run and the optimum responses are shown (PHA, 5μg/ml; Con A, 2μg/ml; LPS, 10μg/ml; bacterin, 1×10^6 cells/ml). The results in stimulation indices (stimulated cpm divided by background cpm) are not mentioned but rather the actual counts per minute because the background levels are very high. I attribute the high background counts to the fetal calf serum that was used, which was the same batch of serum used to support the in vitro spleen cell cultures for the PFC responses. Coutinho and Moller (17) have reported that fetal calf serum was mitogenic for both T and B cells. Many investigators have observed that serum

which supports Mishell-Dutton cultures is especially mitogenic (24). Shigi and Mishell (58) have proposed that serum which supports in vitro PFC responses might actually contain some LPS as a contaminant.

Examination of Table 11 reveals that in both experiments the following trends hold. PHA and Con A effectively stimulated spleen cells from the three mice strains. LPS definitely stimulated the Balb/c and F_1 spleen cells. Whether or not the LPS was mitogenic for the C3H/HeJ spleen cells cannot actually be determined because of the high background levels. This leads to three possible phenomena: 1) the C3H/HeJ mice did respond to the LPS at a low but obscured level, 2) if the serum does have an LPS contamination, as suggested by Mishell, the response noted as background might actually be the LPS mitogenic response, 3) the C3H/HeJ spleen cells did not respond mitogenically to LPS but did respond to some other unknown serum mitogen. No conclusions can be reached, but I favor the last explanation that C3H/HeJ spleen cells are not stimulated by LPS or are stimulated to a very low level by the LPS preparation.

Bacterin was mitogenic for the various spleen cells in both experiments. Clearly, the C3H/HeJ cells responded at

magnitudes similar to the Balb/c spleen cells. It has not been proved that the C3H/HeJ mitogenic response to the bacterin was a response to the LPS on the bacterin; however, the LPS moiety is suspected because the bacterin is a T-independent antigen (5,51), and also because C3H/HeJ mice do make in vitro PFC responses specifically to LPS when immunized with the bacterin. As mentioned, much evidence indicates that a mitogenic response precedes an antibody response.

The mitogen studies confirm that C3H/HeJ mice respond normally to PHA and Con A, and do not respond above background levels to LPS. I have shown that Balb/c, C3H/HeJ and F1 spleen and thymus cells give a mitogenic response to E. coli 0113 bacterin, and have proposed that C3H/HeJ mice do respond in mitogenic assays to LPS that has not been extracted.

TABLE X

Mitogenic Response of Balb/c, C3H/HeJ, and F_1 Thymus Cells to PHA, Con A, LPS, and *E. coli* Bacterin

		CPM		
		Balb/c	C3H/HeJ	F_1
Exp.[a]	Background	1,200	570	760
	PHA	11,000	9,500	8,200
	Con A	135,000	100,000	122,000
	LPS	9,800	750	2,600
	Bacterin	14,500	5,000	5,000
Exp. II[a]	Background	475	390	
	PHA	4,900	6,900	
	Con A	220,000	210,000	
	LPS	2,000	480	
	Bacterin	5,500	4,300	

a) PHA, 5μg/ml; Con A, 2μg/ml; LPS, 10μg/ml; Bacterin, 1 X 10^6 cells/ml

TABLE XI

Mitogenic Response of Balb/c, C3H/HeJ, and F_1 Spleen Cells to PHA, Con A, LPS, and *E. coli* Bacterin

		CPM		
		Balb/c	C3H/HeJ	F_1
Exp. I[a]	Background	60,000	38,000	27,000
	PHA	145,000	160,000	130,000
	Con A	195,000	260,000	210,000
	LPS	185,000	34,000	87,000
	Bacterin	130,000	120,000	52,000
Exp. II[a]	Background	53,000	36,000	
	PHA	105,000	97,000	
	Con A	260,000	350,000	
	LPS	190,000	33,000	
	Bacterin	132,000	112,000	

a) PHA, 5μg/ml; Con A, 2μg/ml; LPS, 10μg/ml; Bacterin, 1 X 10^6 cells/ml

DISCUSSION

The C3H/HeJ mouse strain has been previously reported to be unique in its inherent lack of responsiveness to many of the biological properties of LPS. Several recent reports have capitalized upon the inability of this strain to mount mitogenic responses to endotoxin to probe the mechanisms of B cell activation (11,21,52, 59,71,72,73). Investigators reported earlier that: 1) the C3H/HeJ strain did not give a mitogenic response to endotoxin from E. coli or Salmonella spp. but could respond to other B and T cell mitogens (16,59,66,72), 2) LPS did not function in the C3H/HeJ animals as an adjuvant for other antigens (59,61), 3) LPS did not inhibit the induction of tolerance to protein antigens in C3H/HeJ mice (59), 4) LPS induced a very small polyclonal response in C3H/HeJ animals (16,71), 5) the antibody response was reported to be aberrant in that this strain was either a very low or non-LPS responder over a wide dose range (59,71). Lipid A has been shown to be the effector for mitogenic and adjuvant properties of LPS. Therefore, since the C3H/HeJ mice could respond to the O-polysaccharide of LPS and NPP (73), it appeared that the restricted response to LPS was a failure by this strain to

react to lipid A.

Further, mitogenic and cell mixing studies have demonstrated that the anomalous LPS response cannot be attributed to suppression by macrophages or T cells, lack of macrophage or T cell function, decreases in the number of B cells, altered proportions of T and B cells, or diminished binding of LPS to C3H/HeJ spleen cells. LPS could not be demonstrated to be cytotoxic for the cell cultures at the concentrations used (21,71).

A genetic analysis of the C3H/HeJ response to LPS has indicated that the defect is probably a single gene defect in the B cell which is an autosomal recessive, and not linked to H-2 expression or heavy Ig chain allotype (72).

The interpretation of all the older experiments using LPS and C3H/HeJ mice is that mitogenic, polyclonal, adjuvant effects, and tolerance inhibition are the result of an interaction of lipid A with a receptor on B cells. Further, the experiments with C3H/HeJ mice indicate that the induction of cell division, a mitogenic response, is a sufficient signal by itself to lead to an antibody response.

In this paper, the immune response by C3H/HeJ animals

has been investigated using *in vivo* and *in vitro* techniques to enumerate the number of antibody producing cells in primary and secondary responses to SRBC, Vi, Vi-positive bacterin, PVP, LPS, NPP, and *E. coli* 0113 bacterin. The LPS, NPP, and *E. coli* bacterin was homologous, that is, derived from *E. coli* 0113. Also mitogenic assays were done with PHA, Con A, LPS, and *E. coli* 0113 bacterin. While previous studies have well characterized several aspects of the C3H/HeJ response to LPS, there is very little data about the *in vivo* and *in vitro* antibody responses, especially secondary responses.

Briefly, the *in vivo* data confirm that C3H/HeJ mice are: 1) normal primary responders to the thymus-dependent antigen SRBC, and 2) low primary responders to the thymus-independent antigen LPS. New information established in this report includes: 1) that C3H/HeJ mice can make normal secondary IgG responses to SRBC, 2) that C3H/HeJ mice are able to give primary responses to the thymus-independent antigens Vi, Vi-positive bacterin, and PVP, 3) that C3H/HeJ mice do not make secondary responses to purified *E. coli* 0113 LPS, 4) that C3H/HeJ mice make normal primary responses to *E. coli* 0113 bacterin as

assayed by PFC's produced against SRBC coated with homologous LPS, 5) the C3H/HeJ strain does not appear to generate a secondary response to the E. coli bacterin, 6) that C3H/HeJ animals make a normal primary response to NPP, but NPP does not appear to prime these mice for a secondary response to LPS. Where tested, F_1 animals responded to each antigen in magnitude similar to Balb/c animals.

The in vitro data establishes that C3H/HeJ spleen cells: 1) give near normal primary and secondary responses to SRBC, 2) do not make primary or secondary responses to LPS, 3) might make primary responses to E. coli 0113 bacterin, 4) can make very strong secondary in vitro responses to E. coli 0113 bacterin which are specific for the O-polysaccharide antigenic determinants of homologous 0113 LPS.

The mitogenic assays confirm that C3H/HeJ mice respond normally to PHA and Con A. It was shown that E. coli 0113 bacterin was mitogenic for the C3H/HeJ spleen cells. Whether or not the 0113 LPS was mitogenic could not be determined due to high background levels that might have obscured any response.

While previous studies have well defined the low or absent LPS response in C3H/HeJ animals and subsequently have proposed models for B cell activation, an explanation for the low LPS response itself has remained open. The findings presented in this paper comparing the *in vivo* and *in vitro* responses by C3H/HeJ animals to LPS or *E. coli* bacterin can be interpreted as indicating that this particular mouse strain can indeed make anti-LPS responses and that the genetic B cell defect is recognized when this mouse strain is tested with extracted endotoxin.

The C3H/HeJ strain was shown not to produce primary and secondary antibody responses to LPS either *in vivo* or *in vitro*. However, it was demonstrated that the C3H/HeJ strain does make specific anti-LPS responses to *E. coli* bacterin. *In vivo* strong primary responses were observed and *in vitro* strong secondary responses were demonstrated. The C3H/HeJ may also give primary *in vitro* responses but the interpretation is complicated by the difficulty of eliciting such responses. Primary anti-LPS responses to the *E. coli* bacterin can be explained by the fact that lipid A is not required for the primary

response to the O-polysaccharide antigen (68), and by viewing the bacterin as presenting a relatively unaltered form of LPS. (The role of alterations induced in LPS will be discussed in the following paragraphs.)

The increased anti-LPS secondary response to bacterin observed *in vitro* is interpreted from the premise that an expanded pool of cells, resulting from the primary contact with the antigen, is capable of being triggered by LPS into a secondary response by a second signal. In this case, the second signal is suspected to be lipid A. The lack of a secondary response by mice to other thymus-independent antigens such as SSS III (7,25) or PVP has been suggested by Watson (70) to be the absence of a second signal associated with the antigen. Since it is proposed that lipid A serves as the second signal for the C3H/HeJ secondary anti-LPS response to the *E. coli* bacterin, why is the primary and secondary response to purified LPS so low? Two observations are pertinent. Firstly, a recent paper by Skidmore *et al* (60) has reported that LPS-induced blastogenesis is dependent upon the method used to extract the LPS. It was demonstrated that LPS extracted by the phenol-water method of McIntire *et al* (40), or the

phenol-chloroform-ether method of Galanos et al (19), were "negative" or nonmitogenic in C3H/HeJ cell cultures. These preparations, however, were mitogenic in other mouse strains. LPS extracted by the phenol-water procedure of Westphal (75) was weakly mitogenic for C3H/HeJ cell cultures. "Positive" or mitogenic LPS for C3H/HeJ cultures could be prepared by the butanol method of Morrison and Leive (45), and by the TCA extraction method of Boivin et al (8). The positive LPS preparations described by Skidmore et al (60) were shown by several methods to be LPS and not an unrelated contaminant. Further, in positive LPS preparations, the mitogenic activity resided in the lipid A. When both positive and negative LPS preparations were used together as mitogens it was found that the negative LPS could block any mitogenic response. The LPS which was used in my studies was classified by Skidmore et al (60) as being mildly positive. Secondly, LPS responses could be demonstrated by immunizing the C3H/HeJ mice with E. coli 0113 bacterin and detecting anti-LPS specific antibody producing cells. It is suggested that the bacterin presented a relatively unaltered form of LPS. It might be argued that since the mitogenic data

demonstrates that the bacterin is mitogenic, perhaps other parts of the bacterial cell wall served as the second signal for a secondary response to the O-polysaccharide antigen. It has been reported that the lipoprotein of the outer membrane of E. coli was a B cell mitogen for C3H/HeJ spleen cells (41). The peptidoglycan from gram negative bacteria has also been found to be a mitogen (10,18). Even though other fractions of the bacterin could have been responsible for the mitogenic activity of the bacterin, and hence may have supplied a second signal, the concept that the LPS component participated cannot be ruled out. LPS is proposed as the active moiety in the observed antibody response for the following reasons. Von Eschen (68) has shown that DNP-bacterin can elicit a primary response toward the DNP, however a second challenge with DNP-bacterin results in no secondary response toward the DNP, but rather a definite secondary response to the O-polysaccharide. Further experiments with NPP and lipid A administered simultaneously to a mouse primed with NPP did not result in a secondary NPP response. These results were interpreted to mean that both the antigenic and second signals had to reside on the same molecule. Therefore a

secondary LPS response by mice immunized with bacterin suggests that the C3H/HeJ cells did recognize the lipid A portion of the bacterin LPS and not some other moiety. It would appear then, in light of Skidmore's findings and this present demonstration that C3H/HeJ mice do make anti-LPS antibody responses to E. coli bacterin, that the low response to purified LPS by C3H/HeJ mice is a consequence of alterations induced in the LPS during its extraction. Coutinho and Gronowicz (15) have recently found that C3H/HeJ mice responded equally as well as high LPS responder mice to a thymus-dependent form of E. coli bacterin in primary immunizations.

The failure to find secondary in vivo responses by C3H/HeJ mice to E. coli bacterin is curious. It may be the case that the bacterin (a heat-killed preparation of E. coli cells) does possess a slightly modified endotoxin that the C3H/HeJ B cells can discriminate. If there is a modification, it does not appear to be severe enough to render the C3H/HeJ B cells unable to respond in primary immunizations. However, a second challenge with the bacterin may evoke a more rapid clearance of the antigen and hence a secondary antibody response does not appear.

In vitro the antigen persists and a secondary antibody response can be demonstrated. Some strength for this explanation is found in the original observations by Sultzer (63) that the C3H/HeJ animals exhibited a larger ratio of mononuclear to neutrophilic cells *in vivo* to i.p. inoculations of endotoxin than did other strains of mice.

The ability of C3H/HeJ mice to respond to the thymus-independent antigens Vi, Vi-positive bacterin, and PVP at levels similar to the Balb/c or F_1 animals, suggests that the route of B cell activation for these antigens is not dependent upon any route of LPS activation of B cells. This, however, may not be the case. As mentioned previously, negative LPS could inactivate C3H/HeJ spleen cells from responding to mitogenically active LPS. Further, Glode *et al* (21) have presented data which indicates that LPS which is nonmitogenic is not necessarily inert. C3H/HeJ spleen cells respond to polyinosinic acid in mitogenic assays, but the presence of negative LPS could suppress the mitogenic response to polyinosinic acid. There was an additive mitogenic response in high LPS responder strains to the two mitogens.

Therefore, it is proposed that the anomalous C3H/HeJ

response to endotoxin is the consequence of a genetic defect which limits the response to purified LPS, but does not limit the response to relatively unaltered LPS. This is predicated, in concert with the demonstration of various LPS preparations exhibiting different mitogenic properties, on the observations that primary and secondary responses to LPS cannot be elicited *in vivo* and *in vitro*, but primary and secondary specific anti-LPS responses to *E. coli* bacterin can be elicited *in vivo* and/or *in vitro*.

SUMMARY

The C3H/HeJ mouse strain is refractory to many of the biological properties of LPS. Other investigators have examined the immune response of this strain and have found that various LPS-induced phenomena could not be demonstrated in this animal system. LPS was reported for C3H/HeJ animals not to be: 1) a mitogen, 2) an adjuvant, 3) an inhibitor of tolerance induction, 4) an inducer of a polyclonal response, and 5) a good immunogen. A genetic analysis of this mouse strain concluded that the low LPS response was probably due to a single gene defect expressed on the B cells. The defect was an autosomal recessive trait and not linked to H-2 type or heavy Ig chain allotype. Cellular studies have shown that: 1) suppression by T cells or macrophage, 2) lack of T cell or macrophage function, 3) altered proportions of T or B cells, 4) diminished binding of LPS to the B cells, or 5) LPS cytotoxicity for the cell cultures were not factors responsible for the anomalous LPS response.

Among the several theories for B cell activation, the older C3H/HeJ reports support those hypotheses stating that a mitogenic event is sufficient by itself to initiate B cell maturation to antibody producing cells.

Because there was a paucity of data on the antibody response of this mouse strain and no explanation for the low response itself, this report examines the *in vivo* and *in vitro* antibody responses by the C3H/HeJ mouse to SRBC, Vi, Vi-positive *Citrobacter freundii* bacterin, PVP, LPS, NPP, and *E. coli* 0113 bacterin. Mitogenic studies were done with PHA, Con A, LPS, and *E. coli* bacterin.

It was found that this strain could make primary and secondary responses, both *in vivo* and *in vitro* to the thymus-dependent antigen SRBC. Primary and secondary responses to the thymus-independent antigen purified LPS could not be demonstrated either *in vivo* or *in vitro*. *In vivo* this strain gave normal anti-LPS specific PFC's when immunized with *E. coli* bacterin. No secondary *in vivo* response to the bacterin could be demonstrated and it was proposed that rapid clearance of the antigen did not allow the secondary response to be detected. *In vitro*, the C3H/HeJ spleen cells may have yielded a primary anti-LPS response to *E. coli* bacterin. A very strong anti-LPS secondary response to the bacterin could be elicited *in vitro*.

These results showing responses by C3H/HeJ mice to LPS when immunized with homologous E. coli bacterin either in vivo and/or in vitro, in concert with the recent findings that the method of LPS extraction determines the mitogenicity of the LPS preparation for C3H/HeJ cells, strongly indicates that the low LPS response is a consequence of alterations induced into the LPS during its extraction. The C3H/HeJ genetic defect does not appear to be expressed as a general lack of responsiveness to endotoxin, but rather the defect limits the response to prepared LPS and does not limit the response to endotoxin presented in its native form on bacterin.

APPENDIX

APPENDIX TO RESULTS

Table I

Balb 1° Dir: 44,375; 100,000; 60,000; 50,000;
$\bar{x}$ = 63,594

Fac: 45,625; 61,250; 44,375; 79,375;
$\bar{x}$ = 57,656

C_3H 1° Dir: 121,875; 68,125; 103,750; 59,375;
$\bar{x}$ = 88,281

Fac: 116,250; 66,250; 115,625; 36,875;
$\bar{x}$ = 83,750

F_1 1° Dir: 67,500; 59,700; $\bar{x}$ = 63,600

Balb R° Dir: 3,450; 300; 1,300; 3,350; $\bar{x}$ = 2,100

Fac: 17,125; 14,250; 5,125; 750; $\bar{x}$ = 9,313

C_3H R° Dir: 1,400; 350; 1,050; 500; $\bar{x}$ = 825

Fac: 13,000; 1,700; 25,500; 6,250; $\bar{x}$ = 11,613

Balb 2° Dir: 113,750; 56,875; 127,500; 38,750;
$\bar{x}$ = 84,219

Fac: 1,375,000; 1,500,000; 1,906,250;
893,750; $\bar{x}$ = 1,418,750

C_3H 2° Dir: 41,250; 49,375; 25,000; 15,000;
$\bar{x}$ = 32,656

Fac: 725,000; 731,000; 1,143,750; 981,250;
$\bar{x}$ = 895,313

Table II

Balb, 10^9 cells: 53,000; 56,500; 65,000; $\bar{x}$ = 58,163

C_3H, 10^9 cells: 16,500; 14,250; 18,000; $\bar{x}$ = 16,250

Balb, 1µg Vi : 22,000; 26,000; 32,700; $\bar{x}$ = 26,900

C_3H, 1µg Vi : 5,000; 3,500; 8,750; $\bar{x}$ = 5,750

Balb, 100µg Vi : 2,500; 1,250; $\bar{x}$ = 1,875

C_3H, 100µg Vi : 0; 100; $\bar{x}$ = 50

Table III

Balb, .25µg PVP: 11,500; 12,900; 11,925; 13,275; 13,775; $\bar{x}$ = 12,675

C_3H, .25µg PVP: 13,500; 12,660; $\bar{x}$ = 13,080

F_1, .25µg PVP: 12,780; 11,280; $\bar{x}$ = 12.030

Table IV

Exp. I

Balb, 1^o LPS: 4,800; 2,900; 2,750; 4,375; 3,875; $\bar{x}$ = 3,740

C_3H, 1^o LPS: 925; 300; 25; 425; 475; $\bar{x}$ = 430

Balb, R^o LPS: 0; 50; 200; $\bar{x}$ = 83

C_3H, R^o LPS: 200; 0; 100; $\bar{x}$ = 100

Balb, 2^o LPS: 34,500; 16,500; 14,375; 24,500; 22,500; $\bar{x}$ = 22,475

Table IV (continued)

C_3H, 2^o LPS: 0; 300; 100; 1,050; 0; $\bar{x}$ = 290

Exp. II

Balb, 1^o LPS: 5,000; 7,325; $\bar{x}$ = 6,163

C_3H, 1^o LPS: 650; 425; $\bar{x}$ = 538

F_1, 1^o LPS: 5,188; 7,750; $\bar{x}$ = 6,470

Balb, R^o LPS: 1,150

C_3H, R^o LPS: 325

F_1, R^o LPS: 1,050

Balb, 2^o LPS: 17,200; 25,000; $\bar{x}$ = 21,100

C_3H, 2^o LPS: 2,175; 950; $\bar{x}$ = 1,562

F_1, 2^o LPS: 9,700; 24,375; $\bar{x}$ = 17,038

Table V

Balb, 1^o 0113: 44,700; 35,325; $\bar{x}$ = 40.013 (4 mice, 2 pools)

C_3H, 1^o 0113: 14,575; 19,375; 33,750; 35,000; $\bar{x}$ = 25,675

Balb, R^o 0113: 1,475

C_3H, R^o 0113: 1,325

Balb, 2^o 0113: 306,250; 315,625; $\bar{x}$ = 310,938

C_3H, 2^o 0113: 26,575; 23,750; $\bar{x}$ = 25,163

Table V (Continued)

Balb, 1^{o} 0113: 20,500 (2 mice, one pool)

C_3H, 1^{o} 0113: 33,500 (2 mice, one pool)

Balb, R^{o} 0113: 1,300 (2 mice, one pool)

C_3H, R^{o} 0113: 1,340 (2 mice, one pool)

Balb, 2^{o} 0113: 235,000; 316,260; $\bar{x}$ = 275,630
(4 mice, 2 pools)

C_3H, 2^{o} 0113: 49,000; 67,500; $\bar{x}$ = 58,250
(4 mice, 2 pools)

Table VI

Balb, NPP 1^{o}: 1,260; 1,560; $\bar{x}$ = 1,410

C_3H, NPP 1^{o}: 1,110; 1,290; $\bar{x}$ = 1,200

Balb, LPS 1^{o}: 7,200; 6,000; $\bar{x}$ = 6,600

C_3H, LPS 1^{o}: 330; 450; $\bar{x}$ = 390

Balb, NPP 2^{o}: 2,625; 3,563; $\bar{x}$ = 3,094

C_3H, NPP 2^{o}: 1,213; 1,100; $\bar{x}$ = 1,157

Balb, LPS 2^{o}: 108,438; 123,750; $\bar{x}$ = 116,094

C_3H, LPS 2^{o}: 875; 650; $\bar{x}$ = 763

Balb, NPP 1^{o}/LPS 2^{o}: 86,875; 51,250; $\bar{x}$ = 69,063

C_3H, NPP 1^{o}/LPS 2^{o}: 550; 500; $\bar{x}$ = 525

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