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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Approved:

Norman D. Reed
Chairperson, Graduate Committee

William G. Walter
Head, Major Department

Henry L. Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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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