MONTANA STATE UNIVERSITY

The influence of lipopolysaccharide (LPS) on cellular activities in LPS-unresponsive C3H/HeJ mice
by Patricia Anne Nelson Rampy

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
in Microbiology
Montana State University
© Copyright by Patricia Anne Nelson Rampy (1976)

Abstract:
It has been previously reported that LPS-unresponsive C3H/HeJ mice are refractory to LPS at the B lymphocyte level. The current study was undertaken to determine if other LPS-influenced cellular activities were also defective in these mice. Utilizing adult CBA/J and C3H/HeJ mice as spleen donors, Graft-versus-Host (GVH) reactions were induced in Balb/c neonates. Spleen weight indices failed to distinguish between experimental groups so mortality by day 30 was used as the criterion of induction of GVH disease. Prior LPS treatment of CBA/J adults decreases the ability of their spleen cells to cause fatal GVH disease in Balb/c neonates whereas no difference was found between injection of normal or LPS-treated C3H/HeJ spleen cells. Similar observations were found with these cell types using the mouse spleen mixed leukocyte culture system. There was, however, no suppression of ensuing GVH disease in Balb/c neonates after treatment of spleen donors of either CBA/J or C3H/HeJ origin with butanol-extracted LPS, a preparation previously shown to be mitogenic for C3H/HeJ B lymphocytes. In studies on the effects of serum on GVH disease induction by normal cells, it was found that inoculation of normal CBA/J cells with normal CBA/J serum results in a GVH reaction similar to inoculation of the same type of cells in Hank's balanced salt solution while inoculation in LPS-CBA/J serum shows a significant decrease in resulting mortality. With other combinations of normal or LPS-serum with CBA/J or C3H/HeJ cells, the critical factor appears to be the strain source of the serum rather than prior treatment of the serum donors with LPS or not. There was a nonspecific suppressive effect of C3H/HeJ serum on both C3H/HeJ and CBA/J cells. In another system, layering normal spleen cells on adherent cells taken from LPS-treated CBA/J animals results in loss of GVH reactivity of the subsequent nonadherent preparation whereas incubation with adherent cells from LPS-treated C3H/HeJ mice or normal allogeneic adherent cells produces no such decrease in GVH reactivity. In a carbon clearance assay for stimulation of the reticuloendothelial system with LPS, it was found that the rate of phagocytosis is significantly increased in Balb/c and CBA/J mice 72 hours after inoculation of LPS. No stimulation is seen in rate of carbon uptake in the C3H/HeJ animals after treatment with phenol-extracted LPS or with butanol-extracted LPS. Finally, pretreatment of CBA/J or Balb/c mice trypan blue increases susceptibility to the lethal effects of LPS approximately 12 fold. The main conclusion drawn from this research is that, in addition to the previously reported unresponsiveness of C3H/HeJ B lymphocytes, C3H/HeJ macrophages are also refractory to stimulation by LPS.

THE INFLUENCE OF LIPOPOLYSACCHARIDE (LPS) ON
CELLULAR ACTIVITIES IN LPS-UNRESPONSIVE
C3H/HeJ MICE

by

PATRICIA ANNE NELSON RAMPY

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE
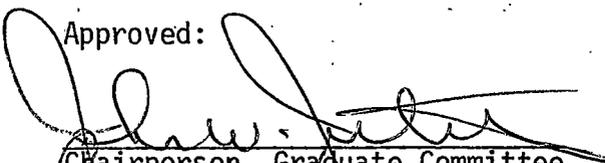
in

Microbiology

Approved:

_____
Chairperson, Graduate Committee

_____
Head, Major Department

_____
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

August, 1976

iii

ACKNOWLEDGMENTS

TABLE OF CONTENTS

v

LIST OF TABLES

ABSTRACT

It has been previously reported that LPS-unresponsive C3H/HeJ mice are refractory to LPS at the B lymphocyte level. The current study was undertaken to determine if other LPS-influenced cellular activities were also defective in these mice. Utilizing adult CBA/J and C3H/HeJ mice as spleen donors, Graft-versus-Host (GVH) reactions were induced in Balb/c neonates. Spleen weight indices failed to distinguish between experimental groups so mortality by day 30 was used as the criterion of induction of GVH disease. Prior LPS treatment of CBA/J adults decreases the ability of their spleen cells to cause fatal GVH disease in Balb/c neonates whereas no difference was found between injection of normal or LPS-treated C3H/HeJ spleen cells. Similar observations were found with these cell types using the mouse spleen mixed leukocyte culture system. There was, however, no suppression of ensuing GVH disease in Balb/c neonates after treatment of spleen donors of either CBA/J or C3H/HeJ origin with butanol-extracted LPS, a preparation previously shown to be mitogenic for C3H/HeJ B lymphocytes. In studies on the effects of serum on GVH disease induction by normal cells, it was found that inoculation of normal CBA/J cells with normal CBA/J serum results in a GVH reaction similar to inoculation of the same type of cells in Hank's balanced salt solution while inoculation in LPS-CBA/J serum shows a significant decrease in resulting mortality. With other combinations of normal or LPS-serum with CBA/J or C3H/HeJ cells, the critical factor appears to be the strain source of the serum rather than prior treatment of the serum donors with LPS or not. There was a nonspecific suppressive effect of C3H/HeJ serum on both C3H/HeJ and CBA/J cells. In another system, layering normal spleen cells on adherent cells taken from LPS-treated CBA/J animals results in loss of GVH reactivity of the subsequent nonadherent preparation whereas incubation with adherent cells from LPS-treated C3H/HeJ mice or normal allogeneic adherent cells produces no such decrease in GVH reactivity. In a carbon clearance assay for stimulation of the reticuloendothelial system with LPS, it was found that the rate of phagocytosis is significantly increased in Balb/c and CBA/J mice 72 hours after inoculation of LPS. No stimulation is seen in rate of carbon uptake in the C3H/HeJ animals after treatment with phenol-extracted LPS or with butanol-extracted LPS. Finally, pretreatment of CBA/J or Balb/c mice trypan blue increases susceptibility to the lethal effects of LPS approximately 12 fold. The main conclusion drawn from this research is that, in addition to the previously reported unresponsiveness of C3H/HeJ B lymphocytes, C3H/HeJ macrophages are also refractory to stimulation by LPS.

INTRODUCTION

The lipopolysaccharide (LPS) fraction of gram-negative bacterial cell walls, also referred to as endotoxin, has been shown to possess a wide variety of biological activities when injected into higher animals. These properties include lethality, toxicity, pyrogenicity, immunogenicity, adjuvanticity, and Schwartzman reactivity (1). LPS is also a B cell (bone marrow-derived lymphocyte) mitogen (2) and a stimulator of polyclonal responses (3).

In 1940, Hill first reported the development of an endotoxin-resistant strain of mice by successive breeding of challenged-resistant generations (4). More recently, Sultzer found the C3H/HeJ mouse strain to be highly resistant to the lethal effects of LPS and to exhibit a different pattern of leukocyte accumulation than is usually seen after intraperitoneal (i.p.) injection of LPS (5). In addition, LPS does not function either as a mitogen (6,7) or an adjuvant in these mice, and the immune response induced is transient (7). Although the genetics of the LPS-nonresponsiveness has not been resolved to date, Sultzer indicated that the lethal effects of LPS and the differential leukocyte accumulation are under polygenic control (8), whereas Watson and Riblet have demonstrated that a single dominant autosomal gene governs mitogenic and immune responsiveness (6). Using cell mixing experiments, it was shown

that the failure of C3H/HeJ spleen cells to exhibit normal responses to LPS was due to nonactivation of B lymphocytes, even though the cells bound LPS as well as B cells from LPS-responsive mouse strains (9). It was suggested that a membrane defect was involved and it has since been reported by Glode et al (10) that the binding of LPS to C3H/HeJ B lymphocyte membranes leads to their inactivation, causing a dose- and time-dependent suppression of B cell stimulation by Poly I, another B cell mitogen.

The elucidation of a B cell membrane defect to account for some of the observed nonresponsiveness to LPS still does not account for the high resistance of the C3H/HeJ to the lethal effects of LPS. Actually, the possibility is raised that similar membrane components may be involved in other LPS interactions in vivo with cell systems not yet explored.

A classic system that has been used to study immunological cellular interactions is the Graft-versus-Host (GVH) reaction. This phenomenon was first described in 1916 by Murphy (11) after he inoculated adult chicken spleen cells onto the chorioallantoic membranes of young chick embryos. The significance of this reaction was not realized until 1953 when Dempster and Simonsen (12) independently expressed the idea that the graft may be capable of mounting an immunological reaction against the host under certain circumstances. A considerable amount of literature has been

published in this area since then, and an extensive review has recently appeared by Grebe and Streilein (13). Since GVH disease is a real problem to immuno-deficient patients who must be reconstituted with lymphoid or lymphoid precursoral tissue, the abrogation of GVH disease has been the specific subject of intensive research. In this regard, Chedid (14) has shown that pretreatment of donor splenocytes in vivo and in vitro with LPS will suppress the GVH reaction. Since then, Thomson and Jutila (15) have reported that it is the adherent population of LPS-treated spleen cells responsible for this suppression, and that these cells interfere in some way with alloantigen-reactive cells in the nonadherent population which cause the GVH disease. It was established by Bona (16) that the macrophage is the cell responsible for processing LPS so it is pertinent to ask if LPS modifies this cell or if it promotes production of a product which mobilizes other cell types.

The objective of the current study was to bring research on GVH disease together with the natural LPS-resistance of the C3H/HeJ mouse strain. It was hoped that such an investigation would reveal new information on the suppression of GVH disease and possibly bring to light additional in vivo mechanisms influenced by LPS-nonresponsiveness in C3H/HeJ mice.

MATERIALS AND METHODS

Mice. Normal five week-old C3H/HeJ and CBA/J male and female
mice were purchased from the Jackson Laboratories, Bar Harbor, Maine,
and maintained in our laboratory in a conventional environment until
use. Conventionally reared Balb/c mice were obtained from our own
stock. All mice were given sterilized Purina 5010C and acidified-
chlorinated water.

Graft-versus-Host Disease. Spleens were removed from adult
C3H/HeJ or CBA/J mice 8-12 weeks of age and placed in 5.0 ml of
Hank's balanced salt solution (BSS) with 5 percent fetal calf serum
(FCS, Grand Island Biological) in Falcon 60 x 15 petri dishes
held on ice. Spleens were gently teased apart into single cell
suspensions, pipetted and passed through an 80 mesh stainless steel
screen. The cells were then counted, centrifuged, and resuspended
in BSS. A total of 2×10^7 spleens cells contained in 0.1 ml were
injected i.p. into Balb/c neonates less than 24 hours old. Cell
preparations of this type are referred to as whole normal (WN)
cells.

Induction of Graft-versus-Host (GVH) disease was measured
initially with a spleen weight assay (17). Mice were sacrificed
on day 10 after injection of adult cells. The spleen index was
taken to equal the spleen weight/body weight of the experimental

neonates divided by the spleen weight/body weight of control neonates injected with BSS. Death between days 7 to 10 or an index of 1.50 or greater was regarded as indicative of an ongoing GVH process. The mice that died before sacrifice were given an arbitrary index of 2.0 for the purpose of calculation of the average indices.

Because spleen indices did not discriminate between experimental groups, death in the interval day 7 to 30 was used as the criterion of GVH disease in all the later work.

Lipopolysaccharide (LPS). LPS was extracted from Escherichia coli 0113, cultures kindly provided by Dr. J. Cutler, Montana State University, and Dr. A. Rudbach, University of Montana, using the hot phenol-water technique of Westphal and Jann (18). The only modification was to incubate the phenol-water mixture at 65C for 30 minutes rather than 15 minutes. The first water extracts from two separate extractions were pooled and dialyzed for four days in double distilled water. Sodium acetate was added to 0.15 M and the LPS was precipitated with 68 percent cold alcohol (final concentration). The precipitate was dissolved in double distilled water, dialyzed for an additional four days and then lyophilized. The potency of the preparation was established by Dr. Kelsey Milner, Rocky Mountain Laboratories, Hamilton, Montana. The results of these bioassays showed that the LPS had a chick embryo LD_{50} of 0.0073 μg and FI_{40} value in rabbits of 0.56 μg (19).

The butanol-extracted LPS (B-LPS) used in two experiments was a generous gift from Dr. David Morrison, Scripps Clinic and Research Foundation, La Jolla, California (20).

Modification of the GVH response by treatment of donor cells.

A. <u>LPS injection of donors</u>. Adult spleen cell donors 8-12 weeks of age were given seven or eight daily injections of 60 µg LPS i.p. Within 24 hours after the last injection, mice were bled for "anti-LPS" serum (LPS-serum) and spleens were removed. Cells (LPS-strain) were harvested in the usual manner and 2×10^7 cells per 0.1 ml were injected into Balb/c neonates less than 24 hours old.

B. <u>Normal or LPS serum given with normal donor cells</u>. Spleen cells were harvested from normal adult mice, counted, pipetted into small tubes, and centrifuged. A total of 2×10^7 cells was resuspended in 0.1 ml of appropriate serum or in Hank's BSS containing 5 percent FCS to serve as controls, allowed to incubate 10 minutes at room temperature, and injected i.p. into Balb/c neonates.

C. <u>"Enriched" cell populations</u>. Spleens were removed from normal or LPS-treated adult mice and placed in 5.0 mls Hank's BSS plus FCS and held on ice. Spleens were gently teased into single cell suspensions, pipetted, passed through an 80 mesh stainless steel screen, and distributed into five 60 x 15 mm plastic Falcon

petri dishes per spleen. After incubation for one hour at 37C, nonadherent (Nonad) cells were decanted, washed two times with BSS, and then adherent (Adh) cells were scraped free with a rubber policeman. If cells were to be injected after this initial separa- tion procedure, cells were washed, counted and 5×10^6 adherent cells per 0.1 ml or 2×10^7 nonadherent cells per 0.1 ml were injected i.p. into Balb/c neonates.

Alternatively, if possible absorptive removal of GVH reactivity by adherent cells was desired, 5×10^6 adherent cells were returned to clean petri dishes and allowed to re-adhere for 30 minutes at 37C. After this time, 2×10^7 normal spleen cells were added to each dish and incubated an additional one and a half to two hours at 37C. Nonadherent cells were removed, centrifuged and the nonadherent content of one dish in 0.1 ml was injected i.p. into Balb/c neonates.

Carbon clearance assay. The procedure followed was modified from the Handbook of Experimental Immunology (21). Normal and LPS- treated mice, four mice per test group, 10-13 weeks of age, were used to assay in vivo phagocytosis. Mice receiving LPS were injected i.v. with 20 µg in 0.1 ml 72 hours before assay. Carbon from Gunther, Pelikan Werke, Hanover, Germany, was diluted in phosphate buffered saline (PBS) to 16 mg/ml. Each animal was weighed and the weight divided by 100 was the amount in millilitres for injection.

Mice were injected i.v. into the tail vein. Twenty-five lambda
of blood were drawn from the retro-orbital plexus at three minute
intervals from time 0 minutes to 15 minutes with heparinized 25
lambda micropipets and placed in four ml of sterile single distilled
water. Optical density (O.D.) was read on a Klett spectrophotometer
using a red filter. The O.D. readings were converted to a
logarithmic scale and plotted versus time. The slope of this line
is the phagocytic coefficient K, which is a measure of the rate of
phagocytosis.

Mixed leukocyte culture. The following protocol for mouse
spleen mixed leukocyte culture was only slightly modified from the
one received from Dr. Fritz Bach, University of Wisconsin, Madison,
Wisconsin. RPMI 1640 (Gibco) culture medium was supplemented with
penicillin, 100 units/ml, and streptomycin, 100 µg/ml. Frozen
human plasma, obtained from a large pool collected at Bozeman
Deaconess Hospital, was thawed and hard-spun at 1650x g for 10
minutes. After heat inactivation at 56C for 30 minutes, the plasma
was hard-spun twice, and added to a final concentration of 5 percent
plasma in RPMI where indicated. To prepare responding and stimu-
lating cells, spleens were removed aseptically into 60 x 15 mm
plastic petri dishes (Falcon #3002) held on ice. Approximately 1.0 ml
of culture medium without plasma was injected into each end of the
splenic capsule to release cells. The spleen was then gently teased

with curved forceps until all the cells had been removed from within the capsule. Cell clumps were separated by repeated pipetting with a 5 ml serological pipet (Falcon #7543). The suspension was passed one time through a 27 gauge needle into a 15 ml centrifuge tube (Falcon #2095) and then centrifuged at 180x g for 10 minutes in the cold. The supernatant was discarded and the cell pellet was resuspended in 0.5 ml of PBS. Red blood cells were lysed by a 10 second exposure to 4.0 ml of sterile, single distilled water, prior to returning the diluent to isotonicity using 0.5 ml of 10X PBS. Cells were centrifuged, supernatant decanted, and pellet resuspended in 5 ml culture medium.

At this point, responding cells were counted in a hemacytometer and diluted to appropriate concentration by adding the amount of plasma needed to bring the final concentration to 5 percent. These cells were then kept cold until added to plates. To the stimulating cells, mitomycin C was added at a concentration of 25 µg per ml cell suspension and mixed thoroughly. The mixture was placed in a 37C water bath for 25 minutes. Cells were then centrifuged, supernatant decanted, and pellet resuspended in medium. Cells were then washed two times by centrifugation and decantation. After final wash, cells were counted and diluted to appropriate concentration, making the final plasma concentration 5 percent.

The responding cells at a concentration of 1×10^6 and 1×10^6 stimulating cells per well were then distributed individually in 0.1 ml volumes to Linbro microtiter plates (Falcon #3040 and #3041) using an 100 lambda Eppendorf pipet with sterile tips. Cultures were incubated at 37C in a humidified 5 percent CO_2, 95 percent air atmosphere incubator. After 72 hours of incubation, 2 µCi ^{3}H-thymidine (specific activity 20 Ci/mmole) was added to each well in 0.05 ml volumes. Sixteen hours later, cultures were precipitated onto Whatman glass fiber filters with saline, 5 percent trichloroacetic acid, and methanol using an Otto Hiller semi-automatic multiple sample precipitator. Uptake of the isotope was determined by placing the filter paper discs containing the cell precipitates into vials with Aquasol (New England Nuclear) and counting in a Beckman LS-100C Liquid Scintillation System.

The data represent the results of triplicate samples from which the sum of the counts of the responding cells alone and the stimulating cells alone have been subtracted.

Effect of trypan blue on LPS LD_{50}. Trypan blue from Kallestad Labs, Inc., Minneapolis, Minnesota, was dissolved in double distilled water and dialyzed against distilled water for 72 hours at 4C. The dialysate was lyophilized and stored in a tightly stoppered bottle at room temperature until used. At time of assay, a fresh sample was resuspended to 10 mg/ml in 0.85 percent saline. The mice

receiving trypan blue were given 4.0 mg i.p. 24 hours before and 1.0 mg i.p. two hours before LPS injection. All animals were then given the specified dose of LPS i.p. and observed for mortality within 72 hours. In most cases, the animals were two to three months old and four or five mice were injected per dose group. The LD_{50} in each assay was computed using the Reed-Muench technique (22).

Statistical analysis. The data presented as means of individual samples were analysed using the student t test. The test was modified for groups of unequal sizes where appropriate. All mortality values were compared using the z test, derived from the normal approximation to the binomial. In all cases, data were tested for significance at the p=0.05 level (23).

RESULTS

GVH assayed using spleen weight indices. To determine whether spleen indices are useful for measuring GVH reactivity, neonatal Balb/c mice were given either 2×10^7 whole normal (WN) or nonadherent (Nonad) cells, or 5×10^6 adherent (Adh) cells from normal CBA/J or C3H/HeJ adults. Other neonates received 2×10^7 whole spleen cells from CBA/J or C3H/HeJ adults given LPS for seven successive days (LPS-). Spleen indices were then calculated on cell or BSS injected mice 10 days post-injection. The number of animals in each group having an index greater than or equal to 1.50 at day 10, and the average index for each test group are shown in Table I. Both the WN CBA/J and Nonad CBA/J spleen preparations caused 100 percent GVH disease in the injected Balb/c neonates as judged by this criterion. Although the average indices of the two groups were different, they were not significantly so. The average index for mice given Adh CBA/J was significantly lower than the index calculated for mice given whole normal spleen cells. The spleen cells from the LPS-CBA/J mice gave spleen weight indices that were essentially the same as nontreated cells.

A wider range of spleen indices was seen with various types of cells from the C3H/HeJ mice. High incidence of GVH disease was caused by both the WN and Nonad-C3H/HeJ cell populations, as indicated

TABLE I

Graft-versus-Host disease in Balb/c neonates assayed using spleen weight indices

Cell Origin[a]	I≥1.50 / Total[b]	Average Index
WN-CBA/J	7 / 7	2.18
Nonad-CBA/J	14 / 14	2.00
Adh-CBA/J	8 / 12	1.69* c
LPS-CBA/J	14 / 17	1.97
WN-C3H/HeJ	29 / 32	2.17
Nonad-C3H/HeJ	16 / 19	1.95
Adh-C3H/HeJ	2 / 11	1.34* +
C3H/HeJ kidney	0 / 8	1.06* + x
LPS-C3H/HeJ	12 / 19	1.97[x o]

[a]2×10^7 WN, Non, or LPS cells or 5×10^6 Adh cells injected i.p. into Balb/c neonates 24 hr old. See Materials and Methods or text for explanation of abbreviations.

[b]Balb/c mice were sacrificed 10 days post-injection. Spleen index equals spleen weight/body weight of experimental neonates divided by spleen weight/body weight of control neonates. Thirty-four BSS controls were included. Those neonates dead between days 7-10 were given an arbitrary index of 2.0. Numerator designates number of mice with an index ≥ 1.50. Denominator designates total mice in each group.

[c]* = significant from WN, + = significant from Nonad, x = significant from Adh, and o = significant from kidney using the student t test for unequal group sizes at p = 0.05 level.

by the number of mice experiencing enlarged spleens and by mean spleen index. With this mouse strain, no significant disease was caused by adherent cells or by kidney cells, the latter being a volume effect control with cells of nonlymphoid origin. The LPS-treatment of C3H/HeJ mice resulted in a mean spleen index that was significantly different only from the adherent and kidney cell preparations.

GVH assayed by mortality. A comparison of GVH reactivity of normal or LPS-treated spleen cells as estimated by mortality is shown in Table II. It is clearly seen that prior LPS treatment of CBA/J animals decreases the ability of their spleen cells to cause fatal GVH disease in Balb/c neonates. In contrast, no difference was found between injection of normal or LPS-treated C3H/HeJ spleen cells.

Mixed leukocyte culture. The mouse spleen mixed leukocyte culture has been reported to be an in vitro correlate to the in vivo Graft-versus-Host process (24). As such, the mixed leukocyte cultures were set up in the same cell combinations as had been used in the GVH assay to determine effect of LPS on GVH reactivity. Analysis of Table III shows that prior LPS treatment of C3H/HeJ mice did not alter the ability of their spleen cells to react against the alloantigens present on the Balb/c lymphocytes as compared to

15

TABLE II

Graft-versus-Host disease in Balb/c mice given spleen cells from
normal and LPS-treated CBA/J and C3H/HeJ mice

Cell Origin[a]	Mortality by Day 30 Post-injection	
	Dead / Total	% Mortality
WN-CBA/J	6 / 7	85.7
LPS-CBA/J	18 / 37	48.6* [b]
WN-C3H/HeJ	15 / 28	53.6
LPS-C3H/HeJ	22 / 37	59.5
BSS Controls	1 / 20	5.0

[a]See Materials and Methods section or text for explanation of abbreviations. All Balb/c neonates received 2×10^7 cells or 0.1 ml BSS i.p.

[b]* = significant difference within group using the z test at the p = 0.05 level.

TABLE III

Mouse spleen mixed leukocyte cultures assaying reactivity of normal or LPS-treated CBA/J and C3H/HeJ leukocytes against mitomycin C-treated (Mc) Balb/c cells[a]

Cell Combinations[b]	Net Average cpm[c]
C3H/HeJ+McBalb/c	4855.42
LPS-C3H/HeJ+McBalb/c	4943.25
CBA/J+McBalb/c	4006.39
LPS-CBA/J+McBalb/c	2174.08

[a]In addition to appropriate controls (not shown), 1×10^6 responding cells in 0.1 ml RPMI and 1×10^6 stimulating cells in 0.1 ml RPMI were added to each well and cultured for 72 hr. Then 2 µCi ^{3}H-thymidine was added, cells were cultured an additional 16 hr, harvested, and counted in a liquid scintillation system.

[b]See Materials and Methods section or text for explanation of abbreviations.

[c]Net average cpm = average of triplicate samples of stimulated culture minus (average counts of responding cells alone + average counts of stimulating cells alone).

counts obtained with normal C3H/HeJ spleen cells. In contrast, reactivity of CBA/J spleen cells was decreased almost two fold by the pretreatment with LPS. These data then support the observations found with similar cell combinations in the GVH system.

GVH reactivity after treatment with butanol-extracted LPS. Since it has been previously reported that the method used to extract LPS affects its ability to evoke a mitogenic response in C3H/HeJ mice (25), it was postulated that method of extraction might likewise affect suppression of GVH by spleens from LPS-treated donors. The data reported in Table IV indicate that no significant difference was found between any of the experimental groups, regardless of strain or previous treatment of spleen donors. Thus, there was no suppression of GVH disease in Balb/c neonates after treatment of spleen donors of either CBA/J or C3H/HeJ origin with butanol-extracted LPS (B-LPS).

Effects of serum on GVH induction by normal cells. It has been recently shown that injection of normal CBA/J spleen cells together with serum from LPS-treated CBA/J animals into Balb/c neonates results in suppression of the ability of those spleen cells to cause fatal GVH disease (26). Table V displays results obtained employing various combinations of sera and cells in a similar system. Inoculation of whole normal CBA/J cells with normal

TABLE IV

Graft-versus-Host reactivity of spleens from mice treated with
butanol-extracted LPS

Cell Origin[a]	Mortality by Day 30 Post-injection	
	Dead / Total	% Mortality
WN-CBA/J	8 / 8	100
B-LPS-CBA/J	10 / 11	90.9[b]
WN-C3H/HeJ	2 / 3	66.7
B-LPS-C3H/HeJ	9 / 11	81.8[b]
BSS Controls	0 / 6	0

[a]See Materials and Methods section or text for an explanation of abbreviations. Each Balb/c neonate received 2×10^7 cells or 0.1 ml BSS i.p.

[b]No statistical difference between experimental groups using the z test at the $p = 0.05$ level.

TABLE V

Effects of serum on Graft-versus-Host reactivity of WN cells when injected together into Balb/c neonates

Cells[a]	Source of Serum[a,b]	Mortality by Day 30 Post-injection	
		Dead / Total	% Mortality
WN-CBA/J	Normal CBA/J	18 / 30	60.0
	LPS-CBA/J	5 / 34	14.7* [c]
	Normal C3H/HeJ	9 / 26	34.6** [d]
	LPS-C3H/HeJ	6 / 16	37.5
	Hank's BSS	12 / 19	63.2
WN-C3H/HeJ	Normal CBA/J	10 / 23	43.5*
	LPS-CBA/J	10 / 22	45.5*
	Normal C3H/HeJ	4 / 22	18.2*
	LPS-C3H/HeJ	4 / 25	16.0*
	Hank's BSS	20 / 26	76.9
BSS Controls	—	1 / 59	1.7

[a]See Materials and Methods or text for explanation of abbreviations.

[b]Each Balb/c neonate received 2×10^7 WN cells in 0.1 ml serum or in 0.1 ml BSS, or BSS alone, i.p. as indicated.

[c]* = significantly different from WN of that strain using the z test at the $p = 0.05$ level.

[d]** = significantly different from WN controls at the $p = 0.06$ level.

CBA/J serum results in a percent GVH disease similar to inoculation of the same type of cells in Hank's balanced salt solution (WN controls). The inoculation of WN cells together with LPS-CBA/J serum yields a significant decrease in mortality. CBA/J cells in a combination with either normal or LPS-C3H/HeJ serum caused mortality similar to each other. Although values are lower than the CBA/J cells in BSS controls, they are not significantly different at the $p = 0.05$ level. When injecting WN C3H/HeJ cells together with serum, the critical factor appears to be the serum source rather than prior treatment of the serum donors with LPS or not. All values reported for combining C3H/HeJ cells with serum are significantly lower than injection of neonates with WN C3H/HeJ cells in BSS. The differences seen using sera from CBA/J versus C3H/HeJ sources are significant at the $p = 0.067$ level.

Removal of GVH reactivity by adherent cells. As described in the Materials and Methods section, spleen cells were allowed to adhere to plastic to separate the adherent and nonadherent cell populations. Adherent cells were then counted and returned to petri dishes and whole normal cells were added. After incubation, it was the nonadherent cells from these combinations that were injected into the neonates. As can be seen in Table VI, the number of mice in each experimental group is very small and the results are taken

TABLE VI

Graft-versus-Host reactivity after layering WN cells on normal or LPS-adherent cells

	Mortality by Day 30 Post-injection	
Cell Origin	Dead / Total	% Mortality
on LPS-CBA/J adh[a,b]:		
WN-CBA/J	1 / 3	33.3
WN-C3H/HeJ	1 / 3	33.3
on LPS-C3H/HeJ Adh:		
WN-CBA/J	3 / 3	100
WN-C3H/HeJ	3 / 3	100
WN-CBA/J on C3H/HeJ Adh	3 / 3	100
WN-C3H/HeJ on CBA/J Adh	3 / 3	100
Nonad LPS-CBA/J[c]	2 / 3	66.7
Nonad LPS-C3H/HeJ	2 / 2	100
WN-CBA/J	5 / 7	71.4
WN-C3H/HeJ	5 / 7	71.4
BSS Controls	0 / 7	0

[a] See Materials and Methods section or text for an explanation of abbreviations.

[b] 2×10^7 WN cells were incubated on 5×10^6 adherent cells for $1\frac{1}{2}$ hr at 37C. The subsequent nonadherent cells were injected i.p. into Balb/c neonates.

[c] 2×10^7 cells injected i.p. into Balb/c neonates.

as indicative of a trend rather than as statistically significant
data. Broadly speaking, therefore, layering normal spleen cells
on adherent cells taken from LPS-treated CBA/J animals results in
loss of GVH reactivity of the subsequent nonadherent preparation
whereas incubation with adherent cells from LPS-treated C3H/HeJ
mice or normal allogeneic adherent cells produced no such decrease
in GVH reactivity. It can also be seen that the nonadherent cells
from both LPS-treated CBA/J and C3H/HeJ are capable of inducing
fatal GVH disease in a manner comparable to normal spleen cells.

Carbon clearance. To examine yet another parameter of
cellular reactivity influenced by LPS treatment, Table VIIa presents
the calculated phagocytic index (K) for Balb/c, CBA/J, and C3H/HeJ
mice with and without prior treatment with 20 µg phenol-extracted
LPS i.v. The rate of phagocytosis is significantly increased in
the Balb/c and CBA/J mice 72 hours after inoculation of LPS. No
stimulation is seen in rate of carbon uptake in the C3H/HeJ animals.

To determine if LPS mitogenic for C3H/HeJ B cells also had
a stimulatory effect on their reticuloendothelial system, a carbon
clearance assay was also conducted on C3H/HeJ mice 72 hours after
i.v. injection of 20 µg butanol-extracted LPS. As can be seen from
Table VIIb, the value of K was not changed from normal by the LPS
injection.

TABLE VII

Rate of phagocytosis in normal or LPS-treated mice measured by a carbon clearance assay[a]

Experiment	Strain	Ave K - Normal	Ave K - LPS-treated
A. phenol-LPS	Balb/c	-0.0375	-0.0590* [b]
	CBA/J	-0.0263	-0.0518*
	C3H/HeJ	-0.0201	-0.0228
B. butanol-LPS	C3H/HeJ	-0.0336	-0.0303

[a]Mice in the LPS groups received 20 µg LPS in 0.1 ml PBS 72 hr before conducting the carbon clearance assay. Each mouse received (body weight divided by 100) ml of 16 mg/ml carbon i.v. and was bled at 3 minute intervals time 0-15 minutes. The slope of a linear regression plot of the log of the optical density readings versus time is the phagocytic index K.

[b]* = LPS-treated is significantly different from normal of same strain using the student t test at the $p = 0.05$ level.

$\underline{LD_{50}}$ of LPS alone or after pretreatment of mice with trypan

blue. Since the C3H/HeJ are known to be resistant to the lethal

effects of LPS, it was necessary to establish the relative LD_{50}

to LPS in the mouse strains employed. As seen in Table VIII, the

CBA/J mice had a LD_{50} of 460 µg and the Balb/c, 750 µg. This

exhibits the well known variation between strains in susceptibility

to LPS. Great resistance is shown, however, by the C3H/HeJ mice

which had a LD_{50} of 9165 µg, a dose 12 times higher than for

Balb/c mice and almost 20 times higher than for the CBA/J mice.

The macrophage is known to be a major processing center for

LPS within the animal so trypan blue was administered to mice prior

to LPS to establish the effect of inactivation of lysosomal enzymes

on susceptibility to LPS. In the case of both the CBA/J and the

Balb/c mice, the LD_{50} was decreased by greater than 12 fold. No

data are shown for the C3H/HeJ mice treated with trypan because

results did not indicate a real influence of the dye on suscepti-

bility to LPS. Rather, this mouse strain exhibited mortality in

eight week old mice treated only with trypan blue, and death in

animals receiving both trypan blue and LPS was random in relation

to dose of LPS received. No such sensitivity to the dye was

observed in C3H/HeJ mice four months of age or older. Work is

now underway to further characterize the reaction of trypan within

the C3H/HeJ reticuloendothelial system.

TABLE VIII

A comparison of LPS LD_{50} in normal or trypan blue pretreated mice

Mouse Strain	LPS LD_{50}[a]	LPS LD_{50} after Trypan Blue[b]
CBA/J	460 µg	37.5 µg
Balb/c	750 µg	60.0 µg
C3H/HeJ	9165 µg	

[a] LD_{50} was calculated using the Reed-Muench technique

[b] Mice in the trypan blue groups were injected i.p. with 4 mg trypan 24 hr before and 1.0 mg trypan blue 2 hr before injection of LPS.

DISCUSSION

Although individual strain differences in reactions to LPS had been recognized before, Sultzer first reported the opposite pattern of leukocyte accumulation after i.p. injection of LPS and the great resistance to the lethal effects of LPS by the C3H/HeJ mouse strain in 1968 (5). Since then, much work has been performed utilizing the C3H/HeJ mice in studies to help elucidate possible cellular and genetic mechanisms in LPS reactivity. In contrast to results found with other mice, LPS does not function as a B cell mitogen (6,7) or an adjuvant in these mice, and the specific immune response elicited is transient (7). By using various cell combinations in vitro and by transfer experiments in vivo, the B lymphocyte of the C3H/HeJ was shown to be the cell responsible for the lack of response. A possible membrane defect was postulated because, although the C3H/HeJ B cell is capable of binding LPS as well as B cells from LPS-responsive mouse strains, no activation results (9). In fact, the binding of LPS to the C3H/HeJ B lymphocyte membranes actually leads to their subsequent inactivation, even causing a dose- and time-dependent suppression of B cell stimulation by another B cell mitogen, poly I (10). The genetics governing the LPS unresponsiveness continues to be an unresolved issue. Sultzer originally reported that the differential leukocyte accumulation

and the high resistance to the lethal effects of LPS was under polygenic control (8). And although Watson and Riblet demonstrated that the mitogenic and immune responsiveness were under control of a single dominant autosomal gene (6), Sultzer has just published a report contending that the lymphocyte activation is governed by a pair of autosomal co-dominant genes (27).

For the most part, C3H/HeJ T lymphocytes and macrophages have been disregarded as far as playing any role in LPS unresponsiveness. In the case of the T cell, this outlook is seemingly justified since T cells do not readily respond to LPS and appear not to interact in LPS responses by B cells, thus leading to the designation of LPS as a thymus-independent antigen (2,28,29). The macrophage, however, can pinocytize LPS and pass the processed molecule to lymphocytes (16). Macrophages are also known to be activated by interaction with LPS (30). Chedid et al have recently reported that C3H/HeJ mice did not exhibit increased nonspecific resistance to infection with Klebsiella pneumoniae after LPS treatment and that, using a tumor cell growth inhibition assay, their macrophages were refractory to in vitro stimulation by LPS (31).

The current study was undertaken to investigate whether other biological reactions influenced by LPS were similarly impaired in the C3H/HeJ mice. Research was conducted in systems involving all three of the traditional immunologically active cell types: the

GVH assay and the mixed leukocyte culture are usually considered tests of T cell function; serum immunoglobulins are considered evidence of B cell function; and adherent cells and the reticuloendothelial system (RES) are related to macrophage cell types, although other cells are obviously involved in the latter populations.

The CBA/J mouse strain was chosen for comparison with reactivity of the C3H/HeJ strain because the CBA/J are LPS-sensitive whereas the C3H/HeJ are not. Also an important consideration is that both strains are H-2^k. Although they are different at minor loci, the mouse major histocompatibility complex is the most important in determining reactions against the alloantigens of the H-2^d Balb/c mice used as targets in the studies.

An increase in spleen weight index is generally regarded as an accurate indication of a GVH reaction. For these studies, however, this assay failed to distinguish between spleen cells from normal or LPS-treated donors of either the CBA/J or the C3H/HeJ mouse strains. Thus, an index indicative of an ongoing GVH process at day 10 was not an accurate predictor of possible eventual survival of similarly treated Balb/c neonates.

On the other hand, employing death by day 30 as the criterion of GVH disease resulted in a clear distinction between experimental groups of the CBA/J strain. As reported by Thomson and Jutila (15),

prior LPS treatment of CBA/J adult spleen donors significantly decreases mortality of injected Balb/c neonates as compared with injection of WN CBA/J cells. This was attributed to activation of the adherent population by the LPS and these cells in turn non-specifically removed the lymphocytes capable of reacting against alloantigens when placed into the immature foreign host. Such a suppressive effect is not evoked by prior LPS treatment of C3H/HeJ spleen donors so the cellular elements, presumably T cells, involved in GVH induction remain unaltered by the presence of LPS.

Similar observations were made from cells reacting in the mixed leukocyte cultures. This would seem to indicate that either the alloantigen reactive cells were present in the LPS-CBA/J system but had been inactivated or that they were removed during laboratory manipulations prior to addition to the mixed cultures. Since many cells are removed in all samples by clumping within tissue elements during centrifugation, the latter possibility is very likely, thus leaving only lymphocytes unable to react to the foreign antigens to be counted and placed in the cultures.

Since method of extraction of LPS from the bacteria affects the ability of the resulting LPS preparation to stimulate B cells from C3H/HeJ mice into a mitogenic response, it was postulated that suppression of GVH reactivity might be similarly affected. Even though butanol-extracted LPS (B-LPS) is mitogenic for B cells

of the C3H/HeJ mice, as well as for LPS-responsive mouse strains (25),

no suppressive effect was exerted on the GVH reactivity of B-LPS

treated C3H/HeJ spleen cells. This indicates that stimulation at

the B cell level does not likewise activate the cells involved in

suppression of allogeneic recognition. The additional evidence of

no decrease in mortality of neonates injected with B-LPS-CBA/J

cells shows that butanol-extraction of LPS affects more of its chemical

properties than had been expected. It should be noted at this point

that, even utilizing 120 μg of B-LPS daily, none of the toxic effects

of phenol-extracted LPS usually shown by the CBA/J animals, such as

diarrhea and some death, were observed. There was also only a slight

splenomegaly after sacrifice, a crude visual observation of inactivity

of the B-LPS preparation even in the LPS-sensitive strain. Recent

work on B-LPS has revealed the presence of a low molecular weight

polypeptide tightly bound to the lipid A, which is removable by

treatment with phenol (32). It is the polypeptide which is responsible

for stimulation of the C3H/HeJ lymphocytes and it is thought that

the peptide blocks a portion of the lipid in much the same manner as

polymixin B does. Therefore, the polypeptide on the lipid A in

B-LPS does not seem to activate the C3H/HeJ adherent cells responsible

for removing GVH reactivity even though mitogenic activity of B cells

is stimulated. Also, the critical portion of a phenol-extracted LPS

molecule responsible for activation of adherent cells in the

LPS-responsive CBA/J mice is not available for reaction on a B-LPS molecule. Thus, with no adherent cell activation, mortality is the same as with untreated normal cells.

The observation by Thomson (26) that serum from LPS-treated CBA/J mice is capable of abrogating a GVH reaction by normal CBA/J cells brings in a humoral factor also involved in suppression. Since CBA/J mice do respond immunologically to LPS as an antigen, serum from mice given seven daily injections of LPS contains anti-LPS antibody (usually with a HA titer of 1:64 or greater, data not shown). It is this anti-LPS component that is proposed to be responsible for the reduced mortality in the Balb/c neonates given both the serum and the WN CBA/J cells. Endotoxemia is a usual phenomenon in animals undergoing a GVH reaction so a possible mechanism for the protective effect could be the prevention of LPS shock within the neonates by the binding of the LPS-serum with LPS during a preliminary GVH reaction. It has been shown (33) that treatment of neonates with antibiotics decreases incidence of GVH disease by just such an endotoxemia prevention mechanism. Normal serum from CBA/J animals given in conjunction with WN CBA/J cells has no abrogating effect. The data presented in the current study include samples repeating those observations. In addition, it was shown that there was no difference in resulting GVH disease by giving either normal or LPS-C3H/HeJ serum. The C3H/HeJ mice

respond poorly and transiently to LPS as an antigen (7) so it would

be expected that no significant antibody element would be produced

under these circumstances (HA titer less than 1:4, data not shown).

It must be noted, however, that just the presence of the C3H/HeJ

serum with the CBA/J cells reduced final mortality markedly, even

though the numbers were not statistically significant at the

$p = 0.05$ level. The question may be raised whether this could be

due to a nonspecific factor that has a suppressive effect or if it

is a phenomenon seen using nonsyngeneic serum. Experiments using

other allogeneic sera or even heterologous sera seem to indicate

that the latter is not the case (data not shown).

With cells from normal C3H/HeJ mice, their injection in

conjunction with any of the sera tested causes a significant decrease

in GVH disease of the Balb/c neonates. Even so, LPS-CBA/J serum does

not further lower the level of reactivity from that obtained from

normal CBA/J serum. This effect cannot then be attributed to an

anti-LPS component found only in the LPS-serum, but rather seems

to be related to the CBA/J origin of the serum. The most surprising

results are found with the combination of C3H/HeJ cells with normal

or LPS serum of syngeneic origin. It is hard to believe that humoral

elements could have such a suppressive effect on the allogeneic

reactivity of their own cells, and no explanation is readily

apparent. Although attempts have been made to compare changes

evoked in reactivity within each mouse strain, it may deserve mention that the drop in reactivity from CBA/J cells in normal CBA/J serum to the level seen of CBA/J cells in the pair of C3H/HeJ sera is comparable to the drop from the reactivity of C3H/HeJ cells in the CBA/J sera down to the values found with C3H/HeJ cells in the C3H/HeJ sera.

The contention that the adherent cell population of LPS-CBA/J mice was responsible for removal of GVH reactive cells (15) was supported and further extended by work in this study. It is seen that LPS-CBA/J adherent cells are capable of reducing mortality not only of WN CBA/J cells but also of WN C3H/HeJ cells. In contrast, reactivity of WN cells of either strain is unaltered by incubation with LPS-C3H/HeJ adherent cells. The important controls of layering WN cells on the adherent cells of the allogeneic strain show that the removal of GVH reactivity is the unique property of the LPS-CBA/J adherent cells. The mechanism of nonspecific absorptive removal and/or cytotoxic effect by activated cells of the adherent population on alloantigen reactive cells is also strengthened because of the suppression of GVH by the allogeneic C3H/HeJ cells as well as the syngeneic CBA/J cells. This is in contrast to the suppression caused by the LPS-CBA/J serum because its effect is only seen with WN cells of CBA/J origin. That T lymphocytes of both strains remain

unaffected by the LPS treatment is seen by comparing reactivity of the nonadherent LPS-cells with mortality caused by WN cells.

As another way of testing whether LPS had any activating effect on macrophage activity within the C3H/HeJ mice, carbon clearance assays were conducted, utilizing increase in rate of phagocytosis as indicative of stimulation. The phagocytic index of both LPS-sensitive mouse strains was significantly increased by pretreatment with LPS. No stimulation of the phagocytic activity by the RES of the C3H/HeJ mice was accomplished by either phenol-extracted LPS or butanol-extracted LPS. Since the latter material is mitogenic for C3H/HeJ B lymphocytes, the low molecular weight polypeptide responsible for the mitogenesis is again seen not to be a stimulatory material for the macrophage. This dichotomy of response has also been recently reported by Chedid (31) in that clearance of bacteria from the blood of C3H/HeJ mice was not increased after treatment with compounds that had been shown to be stimulatory for B cells of the same strain.

While the C3H/HeJ mice are described as being LPS-resistant and other strains as LPS-sensitive, it is necessary to establish what level of sensitivity or resistance is displayed with the particular LPS source and preparation utilized in the study. Of the mouse strains employed here, the CBA/J exhibited the greatest sensitivity with a LPS LD_{50} of 460 µg and the Balb/c were slightly

more resistant with a LD_{50} of 750 μg. Apart from these strain

differences in LPS susceptibility within a limited range, the

C3H/HeJ are truly resistant with a dose of 9165 μg required for

the median lethal effects. This level is 12 times higher than for

the Balb/c and almost 20 times higher than for the CBA/J.

Hibbs (34,35) reported that trypan blue effectively blocks

the activity of macrophages by inactivation of lysosomal enzymes

once the dye has been phagocytosed. As an aside to the other

research on whether or not the C3H/HeJ macrophages were activated

by LPS, a possible mechanism for the C3H/HeJ's great resistance

to the lethal effects of LPS was tested using trypan blue. It

was reasoned that if the C3H/HeJ macrophage was capable of exces-

sive overprocessing and detoxifying LPS within its lysosomes,

inactivation of these organelles should drastically decrease the

LPS LD_{50} of the C3H/HeJ down to a level of similarly treated

sensitive strains. The macrophage indeed plays a substantial role

in trapping and detoxifying LPS because overwhelming the RES with

trypan blue prior to adminstration of the LPS leaves the animals

much more vulnerable to the toxic LPS effects. Thus animals

succumb to much lower doses of LPS than when an intact RES is able

to absorb larger amounts of the LPS before a lethal level is reached

within the animals. The RES blockade could also be combined with

an increased permeability of lysosomes within the reticuloendothelial

cells, which has been reported to increase LPS susceptibility in RES stimulation experiments (36). Indeed, prior injection with trypan blue decreases the LPS LD_{50}'s in both the CBA/J and the Balb/c mice by greater than 12 fold. No real increase in susceptibility to LPS is produced in C3H/HeJ mice by pretreatment with trypan blue. An age-related sensitivity to the dye was revealed and more experiments are planned to possibly elucidate the mechanism of this sensitivity.

In an overview, a few key points have been elucidated in this study. It has been shown that LPS activates CBA/J adherent cells nonspecifically in that suppressive effects are exerted on normal lymphoid cells of allogeneic origin as well as from the same strain, as had been previously reported. In contrast, the abrogation of GVH by LPS-CBA/J serum was effective only on the syngeneic cells in this particular system. There is no activation of a suppressor adherent cell or production of a humoral antibody in the C3H/HeJ that decreases GVH reactivity when compared to normal cells or serum. C3H/HeJ spleen cells also exhibit a lower reactivity when injected in allogeneic sera, whether the donors were pretreated with LPS or not. Notably, C3H/HeJ serum itself possesses some nonspecific suppressive element that decreases activity of its own spleen cells as well as allogeneic cells.

The main theme emerging from this work is that, in addition
to the inactivation of C3H/HeJ B lymphocytes much reported in current
literature, C3H/HeJ macrophages are also refractory to stimulation
by LPS. Even though B cell mitogenesis is seemingly not correlated
with macrophage activation, the nonresponsiveness to the usual
activating action LPS has on macrophages could be due to a membrane
defect in the macrophages similar to the one proposed for B cells.
The possibility that macrophages have actually been inactivated by
contact with LPS would not seem to be the case because the phagocytic
index is not lowered by prior treatment with LPS. It may not be
valid to make this supposition, however, since the duration of
inaction has not been studied and the effect could easily have
diminished by the time of the 72 hour assay. This is certainly
an area that deserves further research attention. General observa-
tions at the cellular level must pave the way for elucidation of
molecular mechanisms of interactions of bacterial LPS within
eukaryotic systems.

REFERENCES

1. Milner, K. C., J. A. Rudbach, and E. Ribi. 1971. General
   Characteristics. In: Microbial Toxins. Vol 4, Edited by
   G. Weinbaum, S. Kadis, and S. J. Ajl. Academic Press, New
   York. P. 1.

2. Andersson, J., G. Möller, and O. Sjöberg. 1972. Selective
   induction of DNA synthesis in T and B lymphocytes. Cell.
   Immunol. 4:381.

3. Andersson, J., O. Sjöberg, and G. Möller. 1972. Induction
   of immunoglobulin and antibody synthesis in vitro by
   lipopolysaccharides. Eur. J. Immunol. 2:349.

4. Hill, A. B., J. M. Hatswell, and W. W. C. Topley. 1940.
   The inheritance of resistance, demonstrated by the develop-
   ment of a strain of mice resistant to experimental inoculation
   with a bacterial endotoxin. J. Hygiene 40:538.

5. Sultzer, B. M. 1968. Genetic control of leucocyte responses
   to endotoxin. Nature 219:1253.

6. Watson, J., and R. Riblet. 1974. Genetic control of responses
   to bacterial lipopolysaccharides in mice. I. Evidence for
   a single gene that influences mitogenic and immunogenic
   responses to lipopolysaccharides. J. Exp. Med. 140:1147.

7. Skidmore, B. J., J. M. Chiller, D. C. Morrison, and W. O.
   Weigle. 1975. Immunologic properties of bacterial
   lipopolysaccharide (LPS): Correlation between the mitogenic,
   adjuvant, and immunogenic activities. J. Immunol. 114:770.

8. Sultzer, B. M. 1972. Genetic control of host responses to
   endotoxin. Infect. Immun. 5:107.

9. Watson, J., and R. Riblet. 1975. Genetic control of responses
   to bacterial lipopolysaccharides in mice. II. A gene that
   influences a membrane component involved in the activation of
   bone marrow-derived lymphocytes by lipopolysaccharides.
   J. Immunol. 114:1462.

10. Glode, L. M., I. Scher, B. Osborne, and D. L. Rosenstreich.
    1976. Cellular mechanism of endotoxin unresponsiveness in
    C3H/HeJ mice. J. Immunol. 116:454.

11. Murphy, J. B. 1916. The effect of adult chicken organ grafts on the chick embryo. J. Exp. Med. 24:1.

12. Billingham, R. and W. Silvers. 1971. The Immunobiology of Transplantation. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. P. 151.

13. Grebe, S. C., and J. W. Streilein. 1976. Graft-versus-Host reactions: A review. Adv. Immunol. 22:119.

14. Chedid, L. 1973. Possible role of endotoxemia during immunologic imbalance. In Bacterial Lipopolysaccharides. Edited by E. H. Kass and S. M. Wolff. University of Chicago Press. P. 104.

15. Thomson, P. D., and J. W. Jutila. 1974. The suppression of Graft-versus-Host disease in mice by endotoxin-treated adherent spleen cells. J. Reticuloendothel. Soc. 16:327.

16. Bona, C. A. 1973. Fate of endotoxin in macrophages: Biological and ultrastructural aspects. In Bacterial Lipopolysaccharides. Edited by E. H. Kass and S. M. Wolff. University of Chicago Press. P. 66.

17. Michie, D. 1973. The biometrics of the spleen weight assay. In Handbook of Experimental Immunology. Second edition. Vol. 2. Edited by D. M. Weir. F. A. Davis Co., Philadelphia, Ch. 30.

18. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides: Extraction with phenol-water and further applications of the procedure. In Methods in Carbohydrate Chemistry. Edited by R. L. Whistler and M. L. Wolfrom. Academic Press, Inc., New York. P. 83.

19. Milner, K. C., and R. A. Finkelstein. 1966. Bioassay of endotoxin: Correlation between pyrogenicity for rabbits and lethality for chick embryos. J. Infect. Dis. 116:529.

20. Morrison, D. C., and L. Leive. 1975. Fractions of lipopolysaccharide from Escherichia coli 0111:B4 prepared by two extraction procedures. J. Biol. Chem. 250:2911.

21. Stuart, A. E., J. A. Habeshaw and A. E. Davidson. 1973.
    Phagocytosis in vitro. In Handbook of Experimental
    Immunology. Second edition. Vol. 2, Edited by D. M. Weir.
    F. A. Davis Co., Philadelphia. Ch. 24.

22. Reed, L. J., and H. Muench. 1938. A simple method of
    estimating fifty per cent end-points. Am. J. Hyg. 27:493.

23. Snedecor, G. W., and W. G. Cochran. 1967. Statistical
    Methods. Sixth edition. Iowa State University Press.
    Ames, Iowa.

24. Elves, M. W. 1969. The mixed leucocyte reaction. An in
    vitro model for the homograft reaction. Transplantation 8:44.

25. Skidmore, B. J., D. C. Morrison, J. M. Chiller, and W. O.
    Weigle. 1975. Immunologic properties of bacterial
    lipopolysaccharide (LPS): II. The unresponsiveness of
    C3H/HeJ mouse spleen cells to LPS-induced mitogenesis is
    dependent on the method used to extract LPS. J. Exp. Med.
    142:1488.

26. Thomson, P. D., J. W. Jutila, and P. A. Rampy. A Mechanism
    for the Suppression of GVH with Endotoxin. Submitted to
    Infect. Immun., 1976.

27. Sultzer, B. M. 1976. Genetic analysis of lymphocyte
    activation by lipopolysaccharide endotoxin. Infect. Immun.
    13:1579.

28. Möller, G., and G. Michael. 1971. Frequency of antigen-
    sensitive cells to thymus-independent antigens. Cell.
    Immunol. 2:309.

29. Andersson, B., and H. Blomgren. 1971. Evidence for thymus-
    independent humoral antibody production in mice against
    polyvinylpyrrolidone and E. coli lipopolysaccharide. Cell.
    Immunol. 2:411.

30. Alexander, P., and R. Evans. 1971. Endotoxin and double
    stranded RNA render macrophages cytotoxic. Nature (London)
    New Biol. 232:76.

31. Chedid, L., M. Parant, C. Damais, F. Parant, D. Juy, and A. Galelli. 1976. Failure of endotoxin to increase non-specific resistance to infection of lipopolysaccharide low-responder mice. Infect. Immun. 13:722.

32. Morrison, D. C., S. J. Betz, and D. M. Jacobs. 1976. Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. J. Exp. Med. in press.

33. Keast, D. 1973. Role of bacterial endotoxin in the Graft-vs-Host syndrome. In Bacterial Lipopolysaccharides. Edited by E. H. Kass and S. M. Wolff. University of Chicago Press. P. 96.

34. Hibbs, J. B. 1975. Activated macrophages as cytotoxic effector cells: I. Inhibition of specific and nonspecific tumor resistance by trypan blue. Transplantation 19:77.

35. Hibbs, J. B. 1975. Activated macrophages as cytotoxic effector cells. II. Requirement for local persistence of inducing antigen. Transplantation 19:81.

36. Lamperle, G. 1966. Effect of RES stimulation on endotoxin shock in mice. Proc. Soc. Exp. Biol. Med. 122:1012.