# Microbiology—1980

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*American Society for Microbiology*
*Washington, D.C.*
1980

1913 I ST., N.W.

WASHINGTON, DC 20006

LIBRARY OF CONGRESS CATALOG CARD NUMBER 75-331066

ISBN 0-914826-23-9

# Introductory Note

Traditionally the major junction of basic microbiology and its practical applications has been in infectious disease, in the response of hosts to infectious agents. Concerning just one aspect of bacterial infection, the proceedings of the recent ASM Conference on Endogenous Mediators in Host Responses to Bacterial Endotoxin, which have pride of place in this volume, survey an astonishing range of studies. Accompanying symposia from the Annual Meeting of the Society discuss some comparative features of the host response to viral infections, including the engrossing phenomenon of interferon induction. Two other important features of virus-host interactions, replication and integration of viral DNA, are also summarized.

Relevant to many of these discussions are newly "emerging" basic science topics, two summarized from additional symposia: one on the development and use of hybridoma technology for the production of monoclonal antibodies and the other on the interaction of DNA with cell membranes.

But, of course, microbiology is practical not only in direct relation to man. All of our students know that every part of the biosphere is dependent on microbial transformations. The burgeoning studies of aquatic microbiology, as summarized from a recent ASM conference, provide many examples. What microbes carry out a particular vital process in the sea? How can microbiology help to solve pollution problems? Only partial answers to these questions are available—and they are enough to indicate why there is always too much material for the volumes in this series.

**David Schlessinger**

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# I. ENDOGENOUS MEDIATORS IN HOST RESPONSES TO BACTERIAL ENDOTOXIN

(from an ASM Conference held 17–19 October 1979 in Norfolk, Va.)

# Introduction

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The ASM Conference on Endogenous Mediators in Host Responses to Bacterial Endotoxin was held in Norfolk, Virginia, on 17–19 October 1979. This conference brought together for the first time leading scientists interested in the varied manifestations of the host response to endotoxic lipopolysaccharides. The meeting was particularly timely since there is an increasing awareness of the presence and significance of soluble mediators in the circulation of the endotoxin-poisoned animal. Collectively, these mediators (lymphocyte-activating factor, glucocorticoid-antagonizing factor, colony-stimulating factor, interferon, tumor necrosis factor, endogenous pyrogen, and prostaglandin) are responsible for the many and varying effects of endotoxin in a susceptible host.

The conference considered the phenotypic expressions of the gene that controls cellular responses to endotoxin and the role of several cell types in the host response to lipopolysaccharides and to the protein moieties associated with lipid A. A major portion of the conference dealt with the problems of isolation and physicochemical characterization of endotoxin-induced mediators derived from serum or produced in cell cultures. The formal presentations and discussions proved extremely helpful in delineating the present stage of knowledge and the uncertainty as to the range of biological activities of the different mediators.

I wish to thank the other members of the organizing committee, L. Joe Berry and David L. Rosenstreich, for their valuable contributions which led to a successful scientific conference. I am particularly indebted to L. Joe Berry and his wife, Tia, for the many hours that they spent on the paper work that goes into the organization of a conference. I would also like to acknowledge with thanks the help given us by Raymond Sarber and David Schlessinger, editor of the *Microbiology* series. Finally, we are appreciative of the support of the National Institute of Dental Research.

# Genetic Control of Endotoxin Sensitivity

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Richard Pfeiffer (1858–1945) gave the term *endotoxin* to the bacterial constituent that was capable of inducing severe pyrogenic and toxic effects in mammals (36). The work of Westphal and Luderitz resulted in the isolation of protein-free lipopolysaccharides (LPS) from many members of the *Enterobacteriaceae* family, and this finally resulted in the finding that the ubiquitous lipid component of LPS, called lipid A, is responsible for many of the endotoxic activities of bacterial endotoxins (9). The diversity of endotoxic responses in mammals has long been a major problem in the study of the mode of action of LPS. It has become a necessary, but not a simple, matter to separate the direct effects of LPS on an LPS-sensitive cell from indirect effects that may result from the subsequent release of mediators which activate cells not themselves sensitive to LPS. The cellular and biochemical reactions that are the basis of the endotoxic response became amenable to genetic analysis when it was observed by Sultzer (29) that there existed a strain of mouse, C3H/HeJ, which was resistant to some of the endotoxic effects of LPS. The finding that the expression of a single locus in C3H/HeJ mice, termed *Lps*, regulates the expression of a number of endotoxic reactions initiated by lipid A (33) allows the use of genetic procedures for segregating direct and indirect LPS effects on cells.

We discuss here the expression of the *Lps* response gene in mice. There exist mouse strains other than C3H/HeJ that appear to exhibit mutations at the *Lps* locus, or possibly in regulatory genes that govern the expression of the *Lps* locus. It is clear that the determination of the *Lps* gene product would greatly facilitate our understanding of the biochemical basis of the initiation of cellular responses to lipid A. We describe several approaches to the analysis of the biochemical nature of the *Lps* gene product.

## THE *Lps* LOCUS

The basic mechanisms by which LPS functions appear to involve the activation of cells such as the cells of the immune system (27, 33, 34), fibroblasts (22), and platelets (19) or the activation of fluid-phase systems such as the complement (10), kinin (15), or coagulation pathways (17). Our approach has been to analyze the effect of LPS on the cells of the immune system and to compare the genetic control of such LPS responses to those observed in other cell types.

Lipid A acts as a specific mitogen for bone marrow-derived (B) lymphocytes in mice (18), and this results in the polyclonal expression of antibody synthesis. C3H/HeJ mice are refractive to the mitogenic and polyclonal effects of LPS (33, 34). This defect appears specific in that other B-cell mitogens such as polyinosinic-polycytidylic acid (poly I:C), dextran sulfate, and a purified protein derivative from tuberculin stimulate mitogenic responses in this strain (34). C3H/HeJ mice support immune responses to the polysaccharide moiety of LPS, which indicates that these antigen-sensitive cells are present. However, all lipid A-induced responses appear defective, including adjuvant effects which are apparent in the immune response to O-polysaccharide antigens of LPS itself as well as to soluble proteins (27, 34).

$F_1$ hybrid progeny from crosses between C3H/HeJ and a number of LPS-responder mice all show intermediate responses to LPS (13). The intermediate response of $F_1$ animals was examined further by autoradiographic studies and has indicated that the number of LPS-responsive cells in $F_1$ cultures is approximately half the number of responsive cells in parent cultures. This finding suggests either a gene dosage or an allelic exclusion phenomenon. In backcross progeny from three $F_1$ strains of mice, (C3H/HeJ × CWB/13) $F_1$, (C3H/HeJ × C57BL/6) $F_1$, and (C3H/HeJ × BALB/c) $F_1$, each backcrossed to the C3H/HeJ parent, responder and nonresponder progeny were observed in a ratio of 1:1. These results suggest that the lack of LPS responsiveness in C3H/HeJ mice is controlled by a single locus, and the alleles at this locus are codominantly expressed (13, 33). The linkage relationships of the polyclonal and adjuvant responses to mitogenic responses in backcross (C3H/HeJ × CWB) $F_1$ × C3H/HeJ mice have also been examined. Mitogenic, polyclonal, and adjuvant responsiveness to LPS all segregated together in the backcross progeny, demonstrating the expression of a single gene which controls each of these responses to LPS (27, 34).

TABLE 1. *Segregation of* Mup-1, Lps, *and* Ps *markers in backcross (C3H/HeJ × C57BL/6By-Ps) $F_1$ × C3H/HeJ mice*[a]

| Region of recombination | Genetic locus | | | No. of mice |
|---|---|---|---|---|
| | *Mup-1* | *Lps* | *Ps* | |
| None | *a* | *b* | + | 15 |
| | *b* | *n* | *Ps* | 10 |
| *Mup-1–Lps* | *a* | *d* | *Ps* | 2 |
| | *b* | *n* | + | 2 |
| *Lps–Ps* | *a* | *n* | *Ps* | 1 |
| | *b* | *d* | + | 1 |
| *Mup-1–Lps–Ps* | *a* | *n* | + | 0 |
| | *b* | *d* | *Ps* | 0 |
| Total | | | | 31 |

[a] The genotypes for *Mup-1*, *Lps*, and *Ps* markers, respectively, are as follows: C3H/HeJ, *a d* +/*a d* +; C57BL/6By-Ps, *b n* Ps/*b n* +; $F_1$, *a d* +/*b n* Ps. Backcross mice were first typed for *Mup-1* and *Ps*, and then spleen cultures were assayed for mitogenic responsiveness to lipopolysaccharide (from reference 31).

The use of a number of recombinant inbred (RI) strains of mice enabled us to extend genetic studies of the defective LPS response gene in C3H/HeJ mice (35). A total of 14 RI strains have been produced by inbreeding, beginning with randomly chosen pairs of mice, from the $F_2$ generation of the cross between C3H/HeJ and C57BL/6J progenitor strains (35). The inheritance of a number of genetic markers was examined in 14 BXH strains. Thirteen of the 14 BXH RI strains exhibited concordant inheritance of the *Mup-1* locus and LPS responsiveness. Only a single strain (BXH-18) possesses a recombinant genotype with respect to LPS responsiveness and *Mup-1*. This degree of concordance is formally significant ($P < 0.01$), suggesting linkage of the two characters (35). A backcross analysis was used to establish that the *Lps* and *Mup-1* loci are genetically linked (31). The data from backcross (C3H/HeJ × C57BL/6J) $F_1$ × C3H/HeJ mice show concordant inheritance of *Mup-1* and *Lps* among 70 backcross mice, indicating linkage. The *Mup-1* locus is linked to the brown coat color (*b*) on chromosome 4 of the mouse (12). Thus, the location of the defective LPS response gene in C3H/HeJ mice is on chromosome 4. We have proposed the locus symbol *Lps* with the mutant allele of C3H/HeJ designated *Lps*$^d$ (defective response) (12).

There are several strains of mice derived from the C57BL/10 strain which are resistant to many of the endotoxic effects of LPS. These include the C57BL/10 ScCR (7) and C57BL/10 ScN (nu/nu) mice (30). Unlike the C3H/HeJ mouse, unresponsiveness to LPS detected in C57BL/10 ScCR mice is inherited as a recessive trait (7), but as shown for C3H/HeJ mice, unresponsiveness is determined by an autosomal gene linked to the *Mup-1* locus on chromosome 4. Since there is no complementation for LPS responsiveness in $F_1$ (C3H/HeJ × C57BL/10 ScCR) hybrid progeny, it appears that C57BL/10 ScCR mice carry a defective allele at the *Lps* locus (7). The distinctive behavior of the defective trait in $F_1$ hybrids between these two nonresponder strains (C3H/HeJ and C57BL/10 ScCR) and several responder strains suggests either that differences exist in the mutation at the *Lps* locus or that there are different effects of background (C57BL/10 or C3H) genes on the expression of the *Lps* locus.

## LOCATION OF *Lps* ON CHROMOSOME 4

We have determined the location of *Lps* on chromosome 4 relative to *Mup-1* and *Ps* in a three-point cross (31). $F_1$ hybrid mice from C3H/HeJ × C57BL/6By-Ps parents were backcrossed to C3H/HeJ. The segregation of phenotypes for *Mup-1*, *Lps*, and *Ps* markers in the backcross progeny is shown in Table 1. The location of the *Lps* locus between the *Mup-1* and *Ps* loci is consistent with the four recombinant phenotypes which were found (Table 1).

## EXPRESSION OF THE *Lps* LOCUS IN NONLYMPHOID CELLS

The major problem in attempting to correlate the genetic control of LPS responses in B lymphocytes to those involving the interaction of LPS with other cell types is the difficulty in performing more than one type of LPS response assay in individual animals. We have examined three nonimmunological responses induced by LPS, utilizing 12 of the recombinant inbred strains of mice derived from C3H/HeJ and C57BL/6J parental strains and a backcross linkage analysis (32). The initiation of hypothermal responses to LPS, and the elevation of serum SAA and colony-stimulating factor levels, are both linked to the expression of the *Lps* locus (32). A summary of these data is presented in Fig. 1.

## DEFECTIVE LPS RESPONSES IN CBA/N MICE

A second strain of mouse expressing a defect in LPS responsiveness is the CBA/N strain. This strain, derived at the National Institutes of Health from the CBA/H strain (3), carries an X-linked mutation which affects B-cell maturation. B cells from these mice fail to express, or express in lower amounts, many of the surface determinants associated with mature B cells (Ia, CR, Mls, Lyb-3, -5, -6, -7) (1, 2, 11, 14, 16, 26, 28).

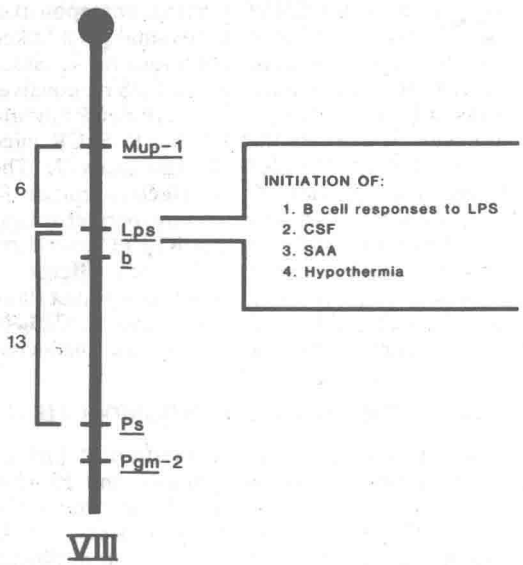


FIG. 1. *Chromosomal location of the* Lps *locus on chromosome 4 (data are from references 31 and 32). LPS, lipopolysaccharide; CSF, colony-stimulating factor.*

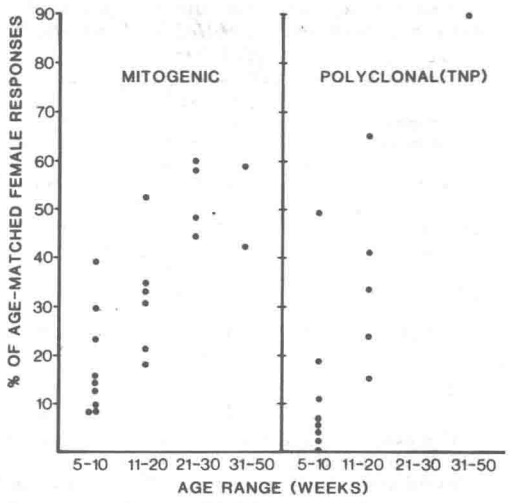


FIG. 2. *Comparative lipopolysaccharide responses in male and female (CBA/N × DBA/2J) $F_1$ hybrids. Each point represents an experiment where the mitogenic or polyclonal responses of an $F_1$ male have been expressed as a percentage of the response of an age-matched $F_1$ female mouse. The assays are described in reference 37.*

In addition, this strain lacks the ability to respond to the group of thymus-independent antigens, termed TI-2, which fail to stimulate responses in neonatal animals (trinitrophenylated[TNP]-Ficoll, type III pneumococcal polysaccharide [SSS-III], poly I:C) (24–26). Responses to thymus-dependent antigens, TI-1 antigens, and polyclonal mitogens (LPS) are present but are also low and often variable (4, 20, 21, 25, 26, 37).

The defective LPS response of CBA/N mice differs in many ways from that of C3H/HeJ mice. One difference is that the defect in the CBA/N mouse is not absolute. Experiments in this laboratory have shown LPS mitogenic and polyclonal responses of spleen cells from CBA/N mice to be low in animals 4 to 6 weeks old, but to increase dramatically with age, approaching the level found for normal mice between 30 and 52 weeks (Fig. 2).

Responses to TI-1 antigens (20) and the thymus-dependent antigen, sheep erythrocytes (SRBC) (37), are also affected by the slower maturation of B cells in mice expressing the X-linked defect. The capacities of normal and defective mice to respond to antigens and mitogens can be compared quantitatively by frequency analysis of B-cell precursors in female and male $F_1$ progeny of a cross between a CBA/N female (xid/xid) and a normal male of another strain (X/Y). This type of analysis shows the defective $F_1$ male to express a lower frequency of LPS- and SRBC-reactive precursors when compared to the phenotypically normal female littermate and shows the increasing responsiveness to SRBC with age to be associated with an increase in the frequency of SRBC-reactive splenic B cells (37; Table 2). Further comparisons of spleen cells from various ages of (CBA/N × DBA/2) $F_1$ mice show the male $F_1$ mice to express a normal percentage of surface immunoglobulin-bearing cells and LPS mitogenic and polyclonal responses equivalent to those of the 5- and 10-week-old $F_1$ females (Table 2). In contrast, the frequency of SRBC-reactive B cells in the 60-week-old male is threefold lower. This indicates that the X-linked mutation may have different degrees of effects on antigen and mitogen responsiveness (Table 2).

TABLE 2. *Effect of age on responses of (CBA/N × DBA/2) $F_1$ male spleen cells*[a]

| Age (wk) | Percent sIg+[b] | Mitogenic responses[c] | Polyclonal responses[c] (TNP) | SRBC-precursor frequencies[d] |
|---|---|---|---|---|
| 5 | 57 | 8 | 4 | 14 |
| 10 | 81 | 40 | 53 | 18 |
| 60 | 96 | 100 | 90 | 42 |

[a] Data taken from reference 37.
[b] Percentage of surface immunoglobulin-bearing cells.
[c] Percentage of 10-week-old female response.
[d] $F_1$ female sheep erythrocyte (SRBC)precursor frequency: 1:12,000.

Two additional characteristics of the defective CBA/N LPS response which distinguish it from that of the C3H/HeJ mice are the limitation of the defect to the B-cell compartment of the immune system and the mapping of the defect to the X chromosome. T-cell and macrophage function in the CBA/N mouse is normal (23), and macrophages from $F_1$ male mice showing no LPS mitogenic response give prostaglandin $E_2$ production, lymphocyte-activating factor production, and killing in response to LPS stimulation which is equivalent to that of the $F_1$ female (21).

The capacity of both macrophages and B cells to respond to LPS maps to chromosome 4, and this is normal in the CBA/N mouse. But the expression of LPS responsiveness in the B cell is controlled or directed by a gene(s) on the X chromosome which affects the general maturation of all B cells, and this gene is defective in the CBA/N mouse. Since lymphoid and nonlymphoid responses to LPS are controlled by the *Lps* locus, the CBA/N strain provides a tool for examining the contribution of LPS-induced B-cell responses to the variety of reactions that accompany endotoxic responses.

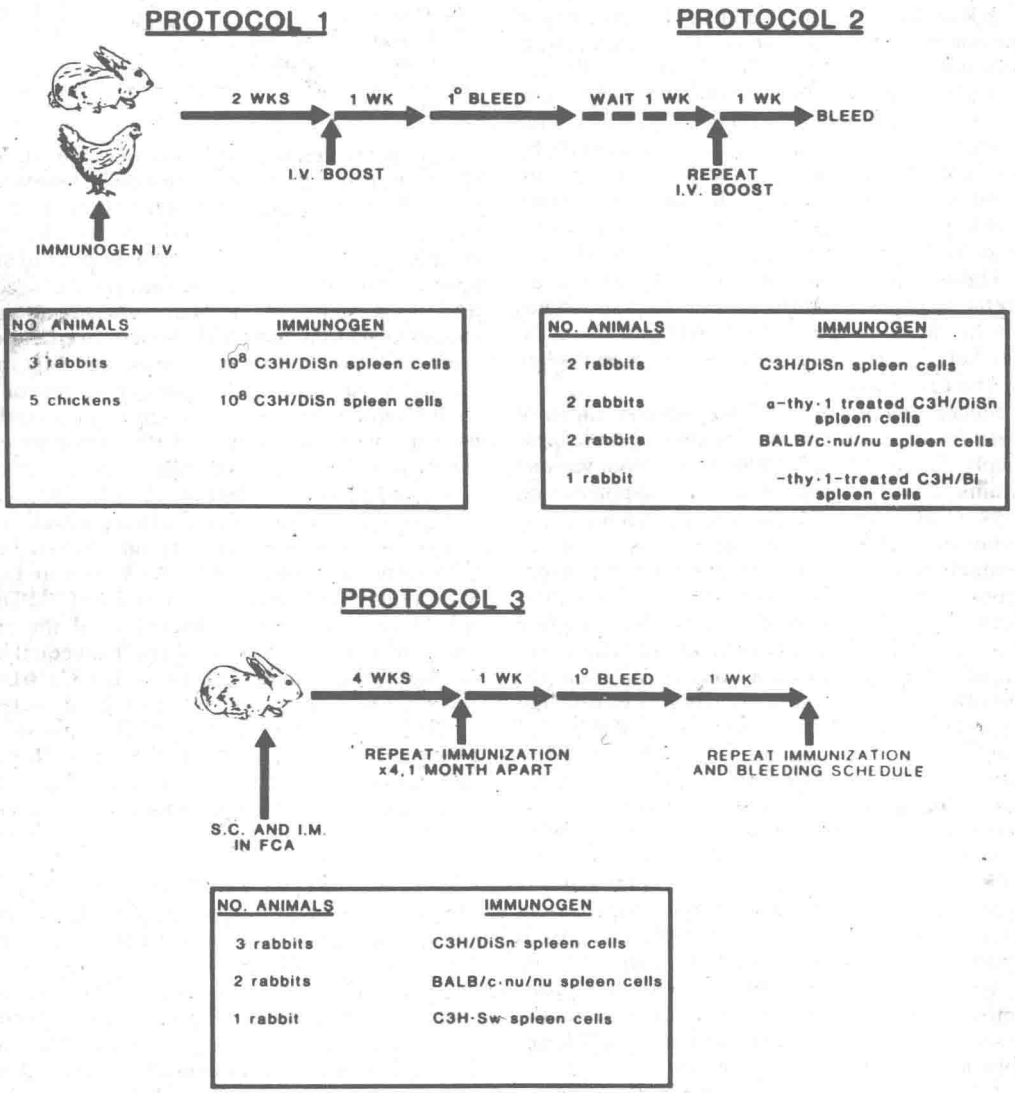


FIG. 3. *Protocols followed in an attempt to prepare antisera specific for a lipopolysaccharide receptor. I.V., intravenous; S.C., subcutaneous; I.M., intramuscular.*

## ANTISERA TO AN LPS RECEPTOR

The expression of the *Lps* locus is involved in the initiation of responses to LPS. The possibility that the *Lps* locus determines a cell surface receptor for LPS was considered by Coutinho and co-workers (5, 6, 8). Rabbits were immunized twice with C3H/Tif spleen cells, and the resulting serum was absorbed both in vivo and in vitro on tissue from C3H/HeJ mice (5, 6, 8). The antiserum prepared in this manner was reported to have the following properties. (i) It reacted specifically with a subpopulation of B cells in all mouse strains tested, except C3H/HeJ and C57BL/10 ScCR mice. (ii) B cells from $F_1$ (R × NR) hybrid mice showed half the reactivity of the parental responder strain. (iii) The antiserum was mitogenic for B cells. Binding to B cells was inhibited by LPS and lipid A. (iv) In backcross (C3H/HeJ × C3H/Tif) $F_1$ × C3H/HeJ progeny, the surface determinants recognized by the antisera segregated with LPS responsiveness. (v) The surface determinants recognized by the antisera co-capped with immunoglobulin D in resting lymphocytes (5, 6, 8, 22).

These results are consistent with the interpretation that such antisera contained antibodies to the lipid A-specific receptor on B cells. Further, this receptor appeared to be controlled by the *Lps* locus.

Spleen cells from LPS-responder-strain mice were injected into rabbits by one of three protocols shown in Fig. 3. Spleen cells from various strains of C3H mice which are responsive to LPS (C3H/DiSn, C3H/Bi, and C3H.SW) in addition to BALB/c nu/nu spleen cells served as immunogens. The C3H/Tif strain was not used. Protocol 1 is the method which has been published by Coutinho et al. (5, 6). This method involves a primary intravenous injection followed after 2 weeks by a booster injection and the collection of antiserum 1 week after the final injection. Protocol 2 is an extension of protocol 1 which involves an additional booster injection and bleeding after the initial serum collection. One group of rabbits received multiple injections of spleen cells emulsified in complete Freund adjuvant (protocol 3). In addition, five different chicken antisera were produced by use of protocol 1. As depicted in Table 3, the antisera were absorbed in vitro on multiple aliquots of spleen, thymus, and lymph node single-cell suspensions from C3H/HeJ mice. Alternatively, the antisera were absorbed in C3H/HeJ mice in vivo followed by an in vitro absorption on C3H/HeJ spleen cells if necessary (Fig. 4).

Antisera were tested for binding to spleen cells from C3H/HeJ and LPS-responder strains by immunofluorescence assays and the induction of mitogenic responses in vitro (Table 3). The most sensitive immunofluorescence assay was found to be a triple sandwich technique using the absorbed α-mouse antisera as the first binding step followed by fluorescein-treated antispecies immunoglobulin antisera for the second and third steps. A number of commercially available second and third antisera preparations were tested in addition to those antisera produced by ourselves. Also, mitogenic responses to the immunoglobulin G fraction of absorbed antisera were assayed in LPS-responder and -nonresponder spleen cell cultures by use of culture conditions described previously (13).

TABLE 3. *Assay of rabbit α-C3H/DiSn spleen cell serum*

| Treatment | Fluorescent-labeled cells | |
|---|---|---|
| | C3H/HeJ | C3H/DiSn |
| Control | 3 | 5 |
| No absorptions | 100 | 100 |
| In vitro absorptions/ml of serum | | |
| 5 C3H/HeJ spleens | 43 | 48 |
| 6 C3H/HeJ spleens | 38 | 34 |
| 7 C3H/HeJ spleens | 39 | 34 |
| 8 C3H/HeJ spleens | 18 | 20 |
| 9 C3H/HeJ spleens | 4 | 7 |
| In vivo absorption | 50 | 54 |
| In vivo absorption followed by in vitro absorption | 0 | 0 |

All of the unabsorbed antisera have high titers of fluorescent binding activity on C3H/HeJ and LPS-responder spleen cells. As shown in Table 3 for a rabbit antiserum raised against C3H/DiSn spleen cells, sequential absorption of the antiserum on C3H/HeJ cells resulted in an equal loss of fluorescence binding activity for C3H/DiSn and C3H/HeJ spleen cells. All of the detectable antibody against C3H/HeJ and C3H/DiSn spleen cells can be absorbed on C3H/HeJ cells. None of the antisera prepared as described in Fig. 3 has been found to bind LPS-responder spleen cells preferentially as determined by fluorescence or mitogenic assays.

To summarize, we have found that rabbit and chicken antisera produced against LPS-responsive spleen cells and absorbed on C3H/HeJ tissues do not differentially recognize a structure on LPS-responsive cells. The question arises concerning the variables in our antiserum production and assay system which differ from those of Coutinho and co-workers (5, 6, 8). Theoretically, an antiserum raised against any LPS-responsive spleen cell population and absorbed on C3H/HeJ tissues should bind only cell surface anti-

**ABSORPTION PROCEDURE**

**In Vivo**

1. Immunoglobulin fraction made by $(NH_4)_2SO_4$ precipitation procedures
2. Inject 1.0 ml intraperitoneally per C3H/HeJ mouse
3. Collect mouse serum after 6 h
4. 50% $(HN_4)_2SO_4$ cut

**In Vitro**

1. Single-cell suspension of spleen, lymph node, and thymus from a C3H/HeJ mouse
2. Absorb whole sera for 1 h at 4°C

**ASSAY PROCEDURES**

**Flourescent Binding Assay (Triple Sandwich)**

1. Absorbed rabbit antiserum
   ↓
   Fl-sheep α-rabbit immunoglobulin
   ↓
   Fl-rabbit α-sheep immunoglobulin
   or
2. Absorbed chicken antiserum
   ↓
   Fl-rabbit α-chicken immunoglobulin
   ↓
   Fl-sheep α-rabbit immunoglobulin

**Mitogenic Assay**

1. DEAE-purified immunoglobulin G fraction
2. Twofold serial dilutions 1:2 to 1:256 into microtiter cultures
3. Label with [$^3$H]thymidine after 48 and 72 h

FIG. 4. *Absorption procedures for rabbit or chicken α-mouse antisera and assay procedures for the absorbed sera.*

gens which are related to LPS responsiveness when tested on the normal parent strain, C3H/DiSn, from which the C3H/HeJ strain was derived. However, specific antiserum production often involves variables which are difficult to define. The most apparent differences between our system and that of Coutinho et al. include the use of different C3H strains as immunogens, a different rabbit population, and different immunofluorescence reagents.

## DISCUSSION

The question arises as to the nature and specificity of the rabbit antisera described by Coutinho and co-workers (5, 6, 8). It is important to point out that the use of these antisera resulted in the identification of the C57BL/10 ScCR strains as LPS nonresponders (5–8). Also, the cell surface determinants expressed by B cells that these antisera recognize appear to segregate in backcross progeny with LPS responsiveness (6). We believe that it is extremely important to determine whether these antisera recognize a cell surface receptor for lipid A.

It is of major interest that there is a region on chromosome 4 that contains loci which control the expression of the Lyb-2, Lyb-4, and Lyb-6 antigens found on B cells (14). Since differences in the alleles expressed at these loci are observed in different C3H and C57BL strains, it is important to exclude the possibility that rabbit antisera specific for putative "LPS receptor" determinants are not binding to Lyb or other B-cell surface antigens.

The expression of the *Lps* regulates B-cell responses to lipid A and does not markedly affect responses to other B-cell mitogens (34). Therefore, it is a reasonable assumption that the *Lps* locus is involved in the early events associated with cell activation by LPS. As discussed, the *Lps* locus may contain structural genes encoding cellular receptors for lipid A. However, there are several other possibilities. The *Lps* locus may also encode for genes that influence the expression of receptors for lipid A or for genes that are involved in the expression of cellular components required to convert the binding event into a biochemical signal. Understanding the biochemical expression of the *Lps* locus is necessary for furthering the solution of the problem surrounding the control of endotoxic responses in mammals.

**ACKNOWLEDGMENTS**

This work was supported by Public Health Service grant AI-13383 from the National Institute of Allergy and Infectious Diseases and by grant 1-469 from the National Foundation. J.W. is supported by a Research Career Development Award (AI-00182) from the National Institute of Allergy and Infectious Diseases.

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