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Genetic Analysis of Lymphocyte Activation by Lipopolysaccharide Endotoxin

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A genetic study of in vitro lymphocyte blastogenesis by the B-cell mitogen lipopolysaccharide endotoxin has been performed, using both low-responder C_3H/HeJ mice and high-responder CBA/J mice. The crossbreeding of these strains produced F1 progeny that were intermediate in responsiveness. Responder types from the backcross of the F1 to the C_3H/HeJ strain segregated into intermediate- and low-responder phenotypes, whereas intermediate- and high-responder phenotypes were produced from the backcross of the F1 to the CBA/J strain. The F2 generation consisted of all three responder phenotypes in frequencies that fit the classical Mendelian ratio of 1:2:1, indicating that the mitogenic response to lipopolysaccharide endotoxin in mice is most likely governed by a pair of autosomal co-dominant genes.

A significant number of the responses of mice to lipopolysaccharide endotoxin (LPS) have been found to be strain dependent. Endotoxemia, the extravascular accumulation of leukocytes, antibody production, polyclonal activation, mitogenicity, and adjuvanticity are responses now recognized to be under genetic control (5-7, 10, 16-18, 23, 24, 27-28). The investigations from which this conclusion developed have, for the most part, relied upon the use of a variant strain of mice, C_3H/HeJ. This endotoxin-resistant mouse profoundly differs from other strains in both its in vivo and in vitro cellular responses to low doses of LPS. Such responses stand in contrast to those seen in endotoxin-susceptible strains of mice. For example, the mononuclear cells and neutrophils of C_3H/HeJ mice appear rapidly in the peritoneal cavity after an injection of microgram quantities of LPS, whereas in other strains of mice this reaction is delayed (23). On the other hand, the exposure of splenic lymphocytes to similar quantities of LPS in culture results in blastogenesis of B cells from LPS-susceptible mice, whereas C_3H/HeJ B cells respond poorly, if at all (16, 25, 27). Similarly, specific antibody production and polyclonal activation ordinarily induced by LPS or lipid A are depressed in C_3H/HeJ mice to the extent that these mice are characterized immunologically as low responders (3, 10, 16).

What operates in the C_3H/HeJ strain to account for this abnormal type of responsiveness to LPS is not known as yet, but it is clear that whatever the mechanism, it is under genetic control. The experiments reported here were undertaken to investigate the type of inheritance controlling the response of mouse B cells to the mitogenic effect of LPS. The results provide evidence that this response is most likely governed by a pair of autosomal co-dominant genes.

MATERIALS AND METHODS

Animals. Inbred C_3H/HeJ ($H\text{-}2^k$) mice were originally obtained from Jackson Laboratories (Bar Harbor, Me.) and subsequently bred in our facilities. Mice were housed in plastic cages and kept at an ambient temperature between 72 and 78 F (22.2 and 25.6 C). All mice were given Purina Mouse Breeding Chow and tap water ad lib. Since it has been found that spleen cells from male and female mice respond identically to the mitogenic effect of LPS, both sexes were used for cell cultures when the mice were 2 to 3 months of age. Reciprocal breeding was conducted to produce F1 and F2 progeny. In addition, F1 mice were backcrossed to each parental type.

Cell cultures. The methods used were essentially those previously described (25), except 1×10^6 viable spleen cells were cultured in 1 ml of RPMI 1640 medium (GIBCO, Grand Island, N.Y.). Fetal calf serum was omitted in the genetic studies to avoid any contribution of suppressive or mitogenic factors present in the serum (4). Normal control and mitogenic responses are obtained under such conditions as long as the medium is used within a 2-week period when stored at 4 C. The medium was supplemented with 100 IU of penicillin and 100 μg of streptomycin per ml. Cultures were incubated at 37 C in the presence of 10% CO_2 in air for a period of 72 h. In certain experiments, the removal of adherent phagocytic cells was accomplished by the use of the carbonyl iron method (15).

Measurement of DNA synthesis. The uptake of [^{3}H]thymidine was used to measure the increase of deoxyribonucleic acid (DNA) synthesis in spleen cells cultured with LPS. Eighteen hours before collecting the cells, 1.0 μCi of [^{3}H]thymidine (5.0 Ci/mmol; Schwarz/Mann, Mount Vernon, N.Y.) was added to each tube. The cells were collected on 0.45-μm cellulose ester filters (Millipore Corp., Bedford, Mass.) in a multimanifold sample collector and washed consecutively with 20 ml of cold saline and 10 ml of cold trichloroacetic acid. The filters were dried at 60 C for 1 h and placed in scintillation vials to which 5 ml of Omnifluor (New England Nuclear Corp., Boston, Mass.) was added. Radioactive counts were made in a Beckman LS 250 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The results are expressed as either the mean counts per minute of triplicate cultures or as a stimulation index (SI). The SI is the mean counts per minute of the experimental culture replicates divided by the mean counts per minute of the background control. Replicate values did not differ by more than 5 to 10%.

Transfer of spleen cells. Spleen cells from C_3H/HeJ or CBA/J donors were prepared in the usual manner in RPMI 1640 without fetal calf serum. Syngeneic or allogeneic transfers were performed by injecting 10×10^7 viable spleen cells into the tail veins of recipient mice that had received 650 R of X irradiation 1 to 2 h previously. Spleen cell suspensions were prepared from the surviving recipients approximately 1, 3, and 6 weeks after transfer and tested for responsiveness to B-cell mitogens.

Mitogens. LPS was made from *Escherichia coli* O127:B8 by the phenol-water method (29). ^{14}C-labeled LPS from *E. coli* O55:B5 was obtained from Sven Britton. This material was prepared by the phenol-water method from organisms grown in synthetic medium supplemented with [^{14}C]glucose. The specific activity of the ^{14}C-labeled LPS was 146,480 counts/min per mg. All LPS preparations were at 1 mg/ml in RPMI 1640 and stored at −20 C. Fresh dilutions were made in the culture medium and added to the cell suspension in 0.1-ml volumes at the onset of incubation. PPD-tuberculin (RT32) was obtained as a dried powder from the Statens Serum Institute, Copenhagen. Stock concentrations of 1 mg/ml were made in RPMI 1640 and stored and used as described for LPS.

LPS binding. Spleen cell suspensions were prepared in the usual manner; however, before the addition of ^{14}C-labeled LPS, the suspension was treated with NH_4Cl-tris(hydroxymethyl)aminomethane buffer according to the method of Boyle (1) to preferentially lyse erythrocytes without damaging lymphocytes. The cells were then washed in protein-free culture medium, made up to 10×10^6 viable cells, and incubated with 50 μg of ^{14}C-labeled LPS in a total volume of 1 ml. From preliminary trials, this concentration of LPS was found to provide a workable level of counts and a maximum amount of binding under these conditions, and the proportion of LPS to cells was in the optimal range for DNA synthesis. After various periods of incubation, the cells centrifuged and the supernatant was added to 10 ml of Packard Insta-Gel emulsifier in a scintillation vial. The cells were resuspended in 1.0 ml of protein-free medium and recentrifuged. This washing procedure was done twice more, and the supernatants were collected as above. The washed cell pellet was dissolved in 1.0 ml of 85% formic acid and added to 10 ml of Insta-Gel. All vials were counted immediately; after quench and background correction, the amount of residual ^{14}C-labeled LPS associated with the cells was calculated.

RESULTS

Dose response. DNA synthesis in CBA/J spleen cells as measured by the uptake of [^{3}H]thymidine was stimulated by LPS, but there was no appreciable synthesis in C_3H/HeJ cells, regardless of the concentrations of LPS used (Fig. 1). In other experiments not shown, even 1 mg of this LPS preparation failed to stimulate proliferation in C_3H/HeJ spleen cells. Since C_3H/HeJ mice are highly resistant to the toxic effects of LPS, it is conceivable that macrophages present in the spleen cell suspensions

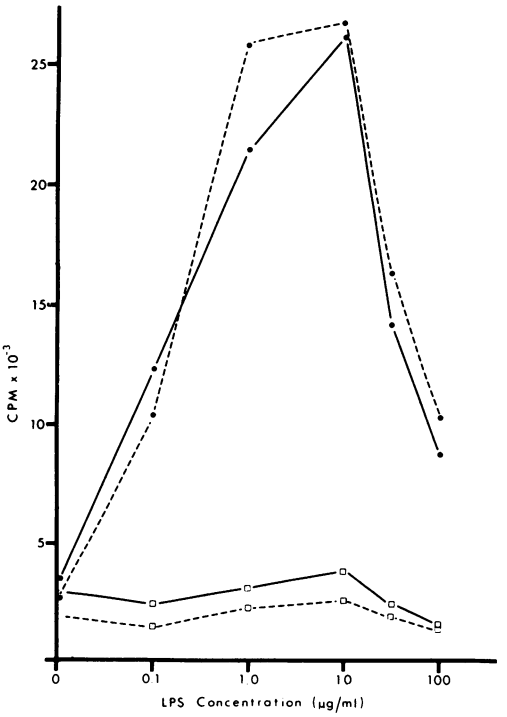

FIG. 1. *Uptake of [^{3}H]thymidine in spleen cells from CBA/J mice (●- - -●), CBA/J spleen cells treated with carbonyl iron (●——●), C_3H/HeJ spleen cells (□- - -□), and C_3H/HeJ spleen cells treated with carbonyl iron (□——□) after exposure to various concentrations of LPS for 72 h of culture. The cells from three mice were pooled for each culture. Each point represents the mean of triplicates.*

could degrade or at least divert LPS from stimulating C_3H/HeJ splenic lymphocytes. However, removal of adherent macrophages and other phagocytic cells by treatment with carbonyl iron powder did not alter the unresponsiveness of C_3H/HeJ cells, nor did such treatment significantly change the dose response curve obtained with CBA/J spleen cells.

Adoptive transfer. To determine whether the unresponsiveness of C_3H/HeJ splenic lymphocytes was due to other host factors that might affect cultured cells, adoptive transfer of spleen cells between C_3H/HeJ and CBA/J mice was attempted. Syngeneic or allogeneic transfer of 10×10^7 spleen cells by the intravenous route was done 1 to 2 h after recipient mice were irradiated. One to six weeks after the transfer, when recipient spleen cells were cultured in the presence of LPS, the response obtained depended on the type of donor cells used in the transfer (Table 1). Spleen cells from CBA/J mice that had received a syngeneic transfer were fully responsive to LPS, whereas cells from surviving C_3H/HeJ mice that had been given CBA/J spleen cells were partially responsive to LPS in culture 1 to 3 weeks after the transfer, and one survivor was fully responsive 6 weeks after the transfer. As expected, no change was seen in recipient C_3H/HeJ mice that had received a syngeneic transfer, but cells from CBA/J mice that had been given C_3H/HeJ spleen cells were unresponsive to LPS. All recipients did respond to PPD (25), another B-cell mitogen to which both mouse strains normally respond, although full responsiveness was not completely attained in the allogeneic transfer. This is most likely a consequence of the difficulties attendant to such transfers.

Genetic analysis. In view of these results, the genetics of the mitogenic response of CBA/J and C_3H/HeJ spleen cells to LPS was analyzed (Table 2). To establish the type of response obtained in hybrid progeny, the data of spleen cell cultures from individual high-responder CBA/J and low-responder C_3H/HeJ mice were grouped according to the range of stimulation indexes obtained with the optimal concentration (10 μg/ml) of LPS that was used throughout. Although the variation in the high-responder cultures is considerable (SI = 9.0 to 35.3), in no case was there any overlap with low-responder values (SI = 1.3 to 3.8). The F1 progeny, with the exception of one animal (SI = 9.6), responded with values intermediate between the two parental classes, indicating no dominance of the high-responder phenotype. However, segregation of the phenotypes did occur in the backcrosses of the F1 with the high- and low-responder types. In both cases, approximately 1:1 ratios were obtained; i.e., about equal numbers of intermediate and high responders were obtained in the backcross to the CBA/J mice; likewise about equal numbers of intermediate- and low-responder mice were obtained in the backcross to the C_3H/HeJ mice. A more extensive survey of F2 mice revealed a segregation into three classes of responses, with the distribution of phenotypes closely approximating a 1:2:1 ratio. An analysis of the observed and expected 1:2:1 F2 frequencies by means of the log likelihood statistic (G test) produced a value of $G = 1.150$ (19). This value, in comparison to a chi-square value of 5.991 at

TABLE 1. *Transfer of mitogen-reactive spleen cells between high- and low-responding mice*

Donor	Recipient	Weeks after transfer	Mean stimulation index of [^{3}H]thymidine uptake by mouse spleen cells in culture	
			PPD	LPS
CBA/J			8.8 ± 0.6[a] (17)[b]	8.7 ± 0.6 (17)
C_3H/HeJ			11.9 ± 0.5 (15)	1.7 ± 0.1 (15)
CBA/J	CBA/J	1	4.5 ± 0.5 (9)	3.5 ± 0.7 (9)
		3	7.4 ± 1.0 (6)	8.6 ± 1.5 (6)
		6	8.0 ± 1.6 (6)	10.5 ± 0.7 (6)
C_3H/HeJ	C_3H/HeJ	1	5.0 ± 1.5 (7)	1.0 ± 0.1 (7)
		3	7.8 ± 0.5 (9)	1.4 ± 0.1 (9)
		6	14.5 (1)	2.0 (1)
CBA/J	C_3H/HeJ	1	4.4 ± 0.6 (9)	3.1 ± 0.6 (9)
		3	4.6 ± 0.7 (7)	3.5 ± 0.4 (7)
		6	5.9 (1)	8.1 (1)
C_3H/HeJ	CBA/J	1	4.3 ± 1.2 (6)	1.2 ± 0.2 (6)
		3	4.9 ± 0.6 (11)	1.1 ± 0.1 (11)
		6	14.1 (1)	1.8 (1)

[a] Standard error of the mean.
[b] Number of mice tested.

TABLE 2. *LPS stimulation of DNA synthesis in spleen cells of high- and low-responding mice and their hybrids*

Strain	No. of mice	Mean SI	Type of response (%)		
			High	Intermediate	Low
CBA/J	50	17.4 (9.0-35.3)[a]	100	0	0
C_3H/HeJ	50	2.5 (1.3-3.8)	0	0	100
F1	28	6.5 (3.9-9.6)	3.6	96.6	0
Backcross (F1 × CBA/J)	28	10.8 (5.2-19.6)	46.4	54.6	0
Backcross (F1 × C_3H/HeJ)	28	3.9 (1.6-8.8)	0	42.9	57.1
F2	70	6.8 (1.3-24.0)	25.7	44.3	30.0

[a] Range of stimulation index.

two degrees of freedom and $P = 0.05$, indicated that the differences between the observed and expected frequencies were not statistically significant.

Binding of LPS. The mechanisms by which the genetic control is exercised remains to be determined. One of the first possibilities considered was that CBA/J and C_3H/HeJ cells differed in their binding capacity for LPS. The results of two binding experiments are presented in Table 3. The amount of ^{14}C-labeled LPS associated with the spleen cells is consistent with data obtained in other antigen systems where animals are unsensitized (22) and with binding data obtained with the mitogens concanavalin A and phytohemagglutinin (21). However, no significant differences appeared between CBA/J and C_3H/HeJ cells in their capacity to bind this preparation of LPS either in the initial or later stages of the culture when activation in high-responder cells has been initiated. Although the possibility exists that the binding of unlabeled LPS might have interfered with the binding of the ^{14}C-labeled LPS, our results are nevertheless in agreement with those of Watson and Riblet, who used a ^{3}H-labeled LPS with C_3HeB/FeJ high-responder cells and C_3H/HeJ low-responder cells (28).

TABLE 3. *Binding of ^{14}C-labeled E. coli O55:B5 LPS to spleen cells from normal mice*

Exposure time (h)	Percent radioactivity bound			
	C_3H/HeJ		CBA/J	
	Expt 1	Expt 2	Expt 1	Expt 2
2	0.83	1.16	0.98	1.10
24	2.02	3.00	1.97	3.01
48	2.00	2.60	2.40	3.20

DISCUSSION

The low responsiveness of C_3H/HeJ lymphocytes to phenol-water-purified LPS has been well documented, and it appears that this deficiency in their B cells is specific. C_3H/HeJ cells proliferate in the presence of PPD, another B-cell mitogen and polyclonal activator (25), as well as respond to other reported B-cell mitogens, including poly(I), poly(I:C), and dextran sulfate (3, 12, 28). In addition, they respond normally to thymus-independent antigens such as pneumococcal polysaccharide type III and the synthetic polypeptide poly(D-Tyr, D-Glu)-poly(D-Pro)-poly(D-Lys) (2, 12). Furthermore, the T cell helper effect functions normally since C_3H/HeJ mice respond to the antigenic stimulus of sheep erythrocytes (B. M. Sultzer, Abstr. Am. Soc. Microbiol., p. 85, M69, 1973) as well as to bovine serum albumin and human gamma globulin (16). The lipid A moiety of LPS does not induce heterophile antibody in these mice (10), nor does it act as a mitogen for their cultured spleen cells (B. M. Sultzer, unpublished data); however, there is a lessened and short-lived antibody response to the complete LPS molecule (16).

From the results of our experiments, this low responsiveness to the mitogenicity of LPS would not appear to be associated with accessory events such as the detoxification or phagocytosis of LPS by macrophages present in endotoxin-resistant C_3H/HeJ spleen cell suspensions. Further, the cell transfer experiment provides evidence that the degree of mitogenic responsiveness is dependent on the genetic constitution of the donor spleen cells, whether CBA/J or C_3H/HeJ, and not to any other host factors, humoral or otherwise, that might contribute to the reactivity of the cells in culture. Similar results also have been reported by Rosenstreich and Glode, who used C_3H/HeJ and C_3H/HeN mice for adoptive transfer (11).

As for the genetic analysis of the responsiveness of the splenic lymphocytes to LPS, the results indicate, first, that the high or low responsiveness is not linked to the H-2^k histocompatibility locus since both CBA/J and C_3H/HeJ are H-2^k. Second, neither is the responsiveness

sex linked, since both sexes in high and low responders and their hybrids behave in the same manner (B. M. Sultzer, unpublished data). These results agree with earlier reports dealing with the response of granulocytes and mononuclear cells to endotoxin (23, 24) and the findings of Watson and Riblet (27), who compared the spleen cells of C_3H/HeJ and CWB mice in their mitogen responsiveness to LPS. Third, with one borderline exception, the F1 progeny of the cross between CBA/J high responders and C_3H/HeJ low responders show intermediate levels of DNA synthesis with no evidence of dominance. Intermediate responses also have been obtained in the F1 hybrids of crosses between C_3H/HeJ and B10:JM or C_3H/TiF high responders (5). In our experiments, backcrosses between (CBA/J × C_3H/HeJ) F1 and CBA/J mice produced segregation into intermediate- and high-responder classes in a ratio of 1:1, whereas backcrosses to the C_3H/HeJ mice produced intermediate- and low-responder progeny also in approximately a 1:1 ratio. These results suggest two co-dominant alleles functioning to control the mitogenic responsiveness to LPS. This tentative conclusion was verified by the interbreeding of F1 mice. The F2 progeny segregated into low-, intermediate-, and high-responder classes closely approximating the Mendelian ratio of 1:2:1. These overall results stand in contrast to those reported by Watson and Riblet (27). F1 hybrid mice of crosses between C_3H/HeJ and DBA/2J or CWB mice were high responders, and backcross progeny from F1 (C_3H/HeJ × CWB) to C_3H/HeJ mice segregated into high and low responders. These workers concluded that a single autosomal dominant gene controls LPS mitogenic responsiveness (27).

The reasons for these different results are not clear. Some considerations might include differences in the preparation of LPS, cultural conditions such as the presence or absence of fetal calf serum in the medium, the number of cells used in the cultures, and the fact that the backcross mice of Watson and Riblet were primed with LPS before being tested for mitogenic responsiveness. It is possible that priming might expand the population of B cells capable of responding to LPS in intermediate animals, so that their cells would respond in vitro to LPS as high responders. Another possibility might be the different high-responder strains used for crossbreeding with the C_3H/HeJ strain; however, it should be noted that CBA/J high responders produce intermediate hybrids in crosses with not only the C_3H/HeJ but also the B10-5M and C_3H/TiF strains (5). Therefore, at least three inbred strains of high-responder mice possess a pair of identical alleles that do not express phenotypic dominance.

If, as suggested from the genetic analysis of our results, a pair of co-dominant genes controls the responsiveness to the mitogenic stimulation of LPS, then it follows that the phenotypic expression of low responsiveness in C_3H/HeJ mice may be determined by a gene product rather than a lack of one due to a recessive genotype. What this gene product might be cannot be deduced from this study; however, since initial LPS binding appears to be similar in low- and high-responder splenic lymphocytes, it seems unlikely that this primary step is genetically controlled. Indeed, LPS binding to mammalian cells has been considered to be nonspecific, possibly due to hydrophobic interaction of the lipid A with membrane lipids (14). Consequently, a secondary structure, whose synthesis is under genetic control, could be the limiting factor.

Whatever this secondary mitogen receptor may be is not apparent as yet, but from a recent report neither C'3 or Fc receptors on murine B cells would appear to be involved in B-cell mitogenesis (9). On the other hand, the type of LPS receptor recently found on human leukocytes might be a candidate (20). If so, this receptor or its murine equivalent in C_3H/HeJ mice may exist in an altered state or may be produced in lesser amounts. As a result, the intermediate mitogenic responsiveness we and others have found may be due to hybrid cells whose surface membranes contain fewer competent receptors or a mixture of competent and incompetent receptors.

Finally, the results of this genetic analysis point to a single gene locus controlling mitogenic responsiveness to LPS. Previous genetic investigations of host responses to LPS in C_3H/HeJ mice have indicated that polygenic inheritance controls both the susceptibility to endotoxemia and the inflammatory cellular response to LPS (24, 26). In the latter case, the C_3H/HeJ mouse could not be characterized as a low responder per se. Rather, the kinetics of the appearance and disappearance of mononuclear cells and neutrophils in the peritoneal cavity of these mice radically differed from those of many other endotoxin-susceptible mouse strains tested under standardized conditions of time and dose.

This type of cellular response as well as resistance to endotoxemia undoubtedly is the end result of a complex series of events including, for instance, the biphasic vascular effects of endotoxin, the interaction with complement, and the release of vasoactive mediators. That this response, therefore, would appear to be

governed by multiple factors is not altogether unexpected.

Although it is clear now that genetic control plays a major role in endotoxin host responses, what gene products are functioning is yet to be determined. In this regard, the C_3H/HeJ strain should continue to serve as an important analytical tool.

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