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LYMPHOID PROCOAGULANT ACTIVITY AND MITOGENESIS IN THE C3H/HEJ MOUSE: DISCORDANT RESPONSE TO LIPOPOLYSACCHARIDE STIMULATION[1]

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Lymphoid cells from the C3H/HeJ mouse respond abnormally to bacterial lipopolysaccharide (LPS) in a number of *in vitro* functions. Abnormalities in both macrophage and lymphocyte populations have been suggested. In the present study, splenic mononuclear cells were isolated from C3H/HeJ, C3H/St, A/J, and BALB/c mice, and analyzed for mitogenesis and induction of procoagulant activity (PCA) by LPS prepared by either phenol or butanol extraction methods. All strains responded vigorously with resultant increased ^{3}H thymidine uptake to Con A and PHA, and all but the C3H/HeJ responded well to butanol or phenol extracted LPS. However, the C3H/HeJ was unresponsive to phenol-extracted LPS and exhibited a poor but significant response to butanol-extracted LPS. In contrast, lymphoid cells from all strains, including the C3H/HeJ, exhibited a marked and equal increase of PCA in response to both phenol- and butanol-extracted LPS by using both splenic and peripheral blood lymphoid cells. The results obtained indicate that although the C3H/HeJ mouse may be unresponsive to a number of LPS-stimulated functions, there is no associated defect in PCA generation, a lymphocyte collaboration-dependent event.

Induction of local fibrin deposition secondary to initiation or amplification of coagulation pathways is characteristically associated with certain forms of immunologic reactions. In both the delayed cutaneous hypersensitivity reaction (1) and the Schwartzman-Santorelli reaction (2), initiation of coagulation and local or disseminated fibrin formation, respectively, are prominent and characteristic features. Induration, a characteristic phenotypic feature of the delayed hypersensitivity reaction, can be abrogated by anticoagulants (3). It is also not observed in the afibrinogenemic patient (4) and can be abolished by carrageenan (5), a macrophage inhibitor. It has been demonstrated that both human and murine lymphoid cells are stimulated by bacterial lipopolysaccharide (LPS) to produce procoagulant activity (PCA)[3] both *in vitro* and *in vivo* (6, 7). It has also recently been demonstrated that murine lymphoid PCA is almost entirely a direct product of the macrophage. As well, lymphocyte collaboration is absolutely required for the induction of macrophage PCA by LPS (8). Information is currently unavailable regarding this pathway of cellular collaboration between lymphocyte and macrophage in the C3H/HeJ mouse, a strain unresponsive in respect to a number of LPS-stimulated functions.

A number of studies (9–11) have demonstrated that murine splenic lymphoid cells of the C3H/HeJ strain are refractory to the mitogenic effects of LPS. The defect in mitogenic response to LPS has been attributed to a mutation at a single gene locus on chromosome four (12). The cause of the defect in the response is, however, far from simple. LPS appears to bind as well to lymphocytes of the C3H/HeJ strain as to other strains. This indicates that a simple receptor deletion cannot be implicated unless only a few of the receptors actually participate in mediation of the biologic response. Alternatively, the receptors could be so modified as to be incapable of functionally translocating the signal in the C3H/HeJ strain. In addition, the capacity of this strain to mount a proliferative response to LPS depends on whether a nonconvalently associated peptide is copurified with the LPS (13). Pure LPS, devoid of this associated peptide, is nonmitogenic for C3H/HeJ lymphocytes, whereas butanol-extracted LPS retains the ability to stimulate mitogenic activity of C3H/HeJ lymphocytes to a low but nonetheless significant degree. This property depends on the presence of the associated small and phenol-dissociable peptide.

In this paper, we analyze the capacity of LPS to induce PCA in C3H/HeJ lymphoid cells. Such a response would clearly indicate separate pathways for the induction of mitogenesis and PCA and indicate that the lymphocytes of this strain are capable of being triggered by LPS, at least to the degree that they then can induce macrophages to produce PCA.

MATERIALS AND METHODS

Lipopolysaccharide. Escherichia coli 0111:B4 were extracted with phenol by the Westphal method (14) to prepare P-LPS, or with butanol to prepare B-LPS as previously described (13). Both purified products were kindly provided by Dr. David Morrison.

Lymphoid cells. A/J and C3H/HeJ mice of 6 to 8 weeks of age were bred in the Research Institute of Scripps Clinic. C3H/

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[3] Abbreviations used in this paper: PCA, procoagulant activity; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HMEM, HEPES-buffered minimum essential medium; P-LPS, phenol-extracted lipopolysaccharide; B-LPS, butanol-extracted lipopolysaccharide.

St and BALB/c mice of similar age were obtained from Strong Laboratories (Sorrento Valley, Calif.). They were sacrificed aseptically, and the spleens were removed. Spleen cells used for PCA analysis were cultured in HEPES- (see Abbreviations) buffered minimal essential medium (H-MEM) consisting of bicarbonate-free minimum essential medium (Flow Laboratories, Los Angeles, Calif.) containing 25 mM HEPES, 2.5 mM NaOH, 100 units penicillin/ml, and 50 μg streptomycin/ml (GIBCO) at pH 7.3. Spleen cells used for mitogenic analysis were placed in RPM1 1640 medium (Flow Laboratories) containing 100 units penicillin/ml and 50 μg streptomycin/ml. Mononuclear cells were isolated by placing 10 ml of a spleen cell suspension containing 7×10^7 cells over 3 ml of Ficoll-Hypaque (density 1.074 g/ml), centrifuging at 22°C and 1400 × G for 12 min, and recovering the mononuclear cells at the interface. The cells were washed twice with either H-MEM or RPM1 1640 and were assayed for viability (98% by trypan blue exclusion).

Peripheral blood mononuclear cells were isolated from both C3H/HeJ and C3H/St mice. Blood was obtained by bleeding from the axilla into heparin and was then centrifuged at 1400 × G for 12 min. The buffy coat was removed and diluted with serum-free medium, layered over Ficoll-Hypaque (density 1.074 g/ml), and centrifuged at 22°C and 1400 × G for 12 min. The mononuclear cells were recovered at the interface.

PCA culture. Peripheral blood mononuclear cells were cultured at 1×10^6 cells/ml, and spleen cells were cultured at 1×10^7/ml in 1 ml volumes in 12 x 75 mm polypropylene tubes (Falcon Plastics, Oxnard, Calif.) in complete medium consisting of 10% heat-inactivated fetal calf serum in minimum essential medium (Flow Laboratories) containing 25 mM HEPES, 0.2 g% sodium bicarbonate, 2 mM glutamine, 100 units penicillin/ml, and 50 μg streptomycin/ml (GIBCO). Cultures were stimulated with 10 μg of either B-LPS or P-LPS, which had been determined from dose titrations to provide the maximal response. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 6 hr, after which they were washed twice with H-MEM and assayed.

Procoagulant activity (PCA). This was determined from a one-stage clotting time assay as previously described (8). Briefly, cells (10^5 to 10^7) were frozen at −20°C and thawed at 37°C, after which they were sonicated in 0.5 ml of H-MEM in 12 x 75 mm tubes with a microtip probe of a Heat-Systems Ultrasonic, Inc., Model W-140 sonicator at a setting of 3 by using two 10-sec bursts each with ice-cooling. After addition of an additional 0.5 ml H-MEM, procoagulant activity was determined from the clotting time at 37°C in recalcified diluted normal human plasma. The assay consisted of 0.1 ml of sample and 0.1 ml of citrated normal human plasma in a 12 x 75 mm tube to which 0.1 ml of 25 mm $CaCl_2$ was added to initiate the assay. The tubes were rocked manually in a 37°C waterbath, and the time (sec) for initiation of clot formation was determined by direct observation. The rabbit thromboplastin standard at 36 mg dry mass/ml (Dade, Miami, Fla.) was assigned a value of 100,000 milliunits. The assay was linear over the range of cell concentrations used and had a precision of 7.0 to 8.3% (coefficient variation). The H-MEM, P-LPS, B-LPS, and complete media containing 10% fetal calf serum alone were without activity.

Mitogenesis. Suspensions of 4×10^5 spleen cells were incubated in microtiter plates (Falcon 3040 microtest II) at 37°C in 5% CO_2 atmosphere in 200 μl of complete medium consisting of 10% heat-inactivated fetal calf serum, RPMI 1640, 2 mM glutamine, 100 units penicillin/ml, and 50 μg streptomycin/ml. Cultures were performed in triplicate and were stimulated with either: a) 0, 1, 5, or 10 μg of either P-LPS or B-LPS; b) serial concentrations of Con A (Miles Laboratories); or c) serial concentrations of phytohemagglutinin (PHA), Calbiochem, La Jolla, Calif.) After 24 hr, 1 μCi ^{3}H-methyl thymidine (2 Ci/mM, Amersham) was added. The cells were incubated for an additional 24 hr and harvested onto fiberglass filters with a Mash II (Multiple Automated Sample Harvester, Microbiological Associates). ^{3}H-methyl thymidine uptake was quantitated by placing the filters in 2 ml of scintillation fluid consisting of 5 gm PPO, 0.1 gm BIS-MSB per 3.785 liter toluene (Fisher Scientific Co.), and counting in a Beckman Scintillation Counter with 47% efficiency. The mean counts of stimulated cultures in triplicate were corrected for uptake of control unstimulated cultures.

Data analysis. All data were statistically analyzed by Student's *t*-test.

RESULTS

Strain-dependent mitogenesis. C3H/HeJ, C3H/St, A/J, and BALB/c spleen cells each demonstrated significant although differing degrees of stimulation of thymidine uptake in response to PHA, Con A, and B-LPS (Fig. 1). Strain differences in the response to B-LPS were clearly evident, with C3H/St cells responding the greatest and the C3H/HeJ cells the least. Dose-response titrations demonstrated equivalent response for each strain over a range of concentrations of B-LPS and maximal responses at 10 μg/ml. When spleen cells were exposed to P-LPS, the spleen cells of C3H/St, BALB/c, and A/J strains responded only slightly less than to B-LPS; however, C3H/HeJ cells were virtually unresponsive (Fig. 1).

Induction of PCA. LPS-stimulated and control splenic lymphoid cells were assayed for PCA. When 5×10^6 murine splenic lymphoid cells were incubated for 6 hr with increasing concentrations of B-LPS, PCA was induced, and the dose-response curves were parallel to that for standard thromboplastin (Fig. 2). Maximal responses were observed at 10 μg B-LPS, as exemplified in Figure 3. The time course of the

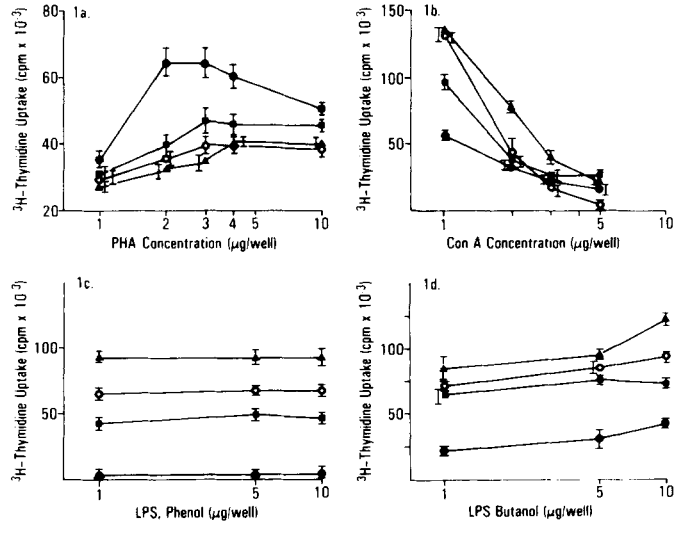

Figure 1. ^{3}H Thymidine uptake in stimulated murine splenic lymphoid cells. (●——●, C3H/HeJ; ▲——▲, C3H/St; O——O, BALB/c; ■——■, A/J). 4×10^5 murine splenic lymphoid cells were incubated with increasing concentrations of a) PHA, b) Con A, c) phenol extracted LPS, and d) butanol extracted LPS for 24 hr, pulsed with ^{3}H thymidine, and harvested and counted. The mean counts of stimulated cultures in triplicate were corrected for uptake of control unstimulated cultures.

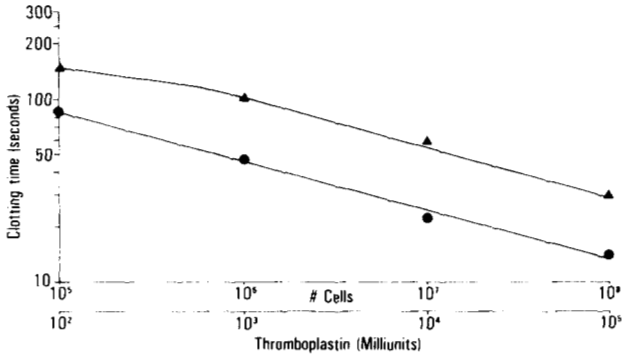

Figure 2. Procoagulant activity generated by rabbit brain thromboplastin standard and LPS-stimulated C3H/HeJ splenic lymphoid cells. ●——●, Log dilutions of rabbit brain thromboplastin standard were prepared and assayed for PCA in a one-stage clotting assay; ▲——▲, 5 × 10^6 C3H/HeJ murine splenic lymphoid cells were stimulated with 10 μg B-LPS and total content PCA was determined for 10^5 to 10^8 cells.

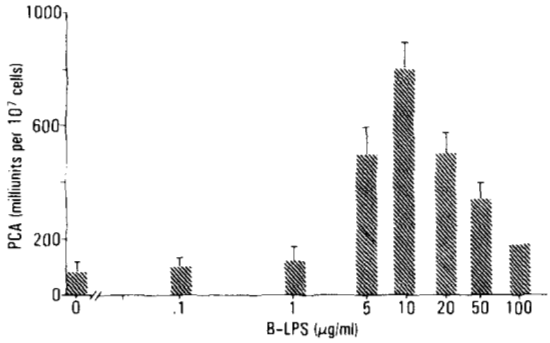

Figure 3. Effect of concentration of lipopolysaccharide on procoagulant activity induction in C3H/St murine spleen cells. 5 × 10^6 murine splenic lymphoid cells were incubated with increasing concentrations of B-LPS (*E. coli* 0111-B4) from 0.1 μg to 10 μg and total content PCA determined.

response was analyzed by using 5 × 10^6 murine lymphoid cells from all four strains, which were exposed to 10 μg B-LPS for varying periods before analysis (Fig. 4A). PCA was maximal after 6 hr incubation without evidence of further stimulation by incubation for longer periods. The time course and dose response curves were equivalent for all four strains, as we have shown previously (8). Basal PCA content was in subsequent experiments determined by assaying 5 × 10^6 lymphoid cells that had been incubated for 6 hr in the absence of LPS and was varied between strains from 130 to 217 milliunits (Table I). There was a comparable stimulation by B-LPS and P-LPS of PCA by using spleen cells from all four strains. Cells of all strains showed an equivalent maximal PCA output of approximately 800 milliunits by using both P-LPS and B-LPS. Specifically, the C3H/HeJ strain responded equally well to both B-LPS and P-LPS with increased generation of PCA that was comparable to the other strains.

In addition, C3H/HeJ peripheral blood mononuclear cells responded equally well to both P-LPS and B-LPS stimulation, with an increase in PCA from 372 ± 183 milliunits basal to 1433 ± 433 milliunits, and 395 ± 110 milliunits basal to 1233 ± 153 milliunits, respectively, per 10^6 mononuclear cells.

DISCUSSION

In the present experiments, cells of the C3H/HeJ strain were examined for the capacity to respond to LPS with resultant amplification of PCA content. As described by others, these cells were mitogenically unresponsive to P-LPS although mildly responsive to the B-LPS. In contrast, both C3H/HeJ spleen cells and peripheral blood mononuclear cells responded in a similar fashion to LPS as did C3H/St and other strains with increased PCA production. We do note, however, that peripheral monocytes generate 10 to 15 times the PCA of splenic macrophages. The exact basis for this difference between the quantity of PCA generated by splenic macrophages compared with peripheral blood monocytes is under investigation.

Bacterial LPS possesses a broad array of biologic properties. It is a mitogen for murine B lymphocytes (15), an adjuvant of antibody formation (16), and as an antigen is highly immunogenic and can elicit a specific antibody response (16). In addition to these properties, it can stimulate the production of PCA by both human and murine lymphoid cells (6–8). We have recently shown by direct analysis in a plaque assay that the murine splenic macrophage is the source of the increased PCA; however, augmentation of PCA content and induction of expression by viable cells seems to have an absolute requirement for lymphocyte collaboration (8). Analyses of these responses in the C3H/HeJ mouse may shed some information regarding the mechanisms, since this strain is selectively responsive to only some of the effects of LPS. It has been suggested that the genetically prescribed defect in the mitogenic response of the C3H/HeJ strain to LPS lies in the B lymphocyte population (10), although there might also be an LPS-related macrophage defect as well. In this respect, Ruco *et al.* (17) have observed that C3H/HeJ macrophages cannot respond in a tumoricidal fashion to LPS. Although the precise nature of the B cell defect has not been elucidated, it appears that the B cell of the C3H/HeJ does possess surface receptors capable of binding LPS (12). Whether a discrete and minor subpopulation of "triggering"

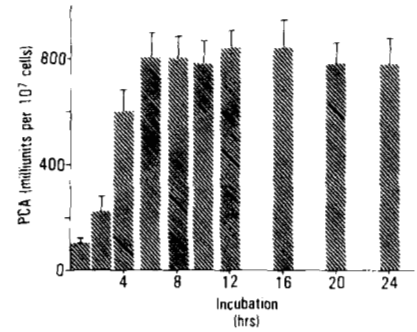

Figure 4. Effect of incubation on procoagulant activity generation in C3H/St splenic murine lymphoid cells in response to LPS stimulation. 5 × 10^6 murine splenic lymphoid cells were incubated with 10 μg B-LPS for 30 min to 24 hr and total content PCA was determined.

TABLE I

Amplification of murine spleen cell procoagulant activity by LPS

Strain	PCA per 10^7 Mononuclear Cells					
	P-LPS			B-LPS		
	Stimulated	Unstimulated	Index[a]	Stimulated	Unstimulated	Index[a]
	Milliunits			*Milliunits*		
C3H/HeJ	783 ± 29	210 ± 20	3.7	724 ± 55	217 ± 17	3.3
C3H/St	810 ± 10	215 ± 5	3.8	830 ± 80	210 ± 20	3.9
BALB/c	810 ± 85	130 ± 50	6.2	740 ± 30	90 ± 10	8.2
A/J	790 ± 100	140 ± 30	5.6	795 ± 65	80 ± 10	9.9

[a] The stimulation index is the ratio of stimulated to unstimulated cells.

receptors are missing or whether these receptors may be functionally defective so as to preclude cell activation is not known.

The present study demonstrates that the macrophage, which is the cellular source of PCA (8), is capable of producing equivalent amounts of PCA in all strains analyzed. This indicates that whatever the genetic defect imposed on the C3H/HeJ strain in respect to other lymphoid functions is, this genetic locus does not restrict the induction of PCA. In addition, since lymphocyte collaboration is essential for amplification of PCA content and viable expression of PCA by the macrophage, it appears that this pathway of collaboration of the LPS-induced lymphocyte with the macrophage must also be intact. Exactly which cells or subsets of cells within the lymphocyte population are required for generation of macrophage PCA is not known at this time. However, studies are presently underway to determine whether, in fact, this process acts through the B cell or some other subset. Although a major difference in time course between PCA induction and mitogenesis might be considered, early induction events in mitogenesis also occur in the time frame within which macrophage PCA is induced. This new evidence that the C3H/HeJ strain can respond normally to LPS in respect to this function indicates that this strain is not refractory to all the effects of LPS as might be anticipated from deletion or complete nonfunction of a single specific receptor. This dichotomy in responses will have to be accommodated in attempts to elucidate the effects of LPS on lymphocytes. Whether it lies in the participation of different lymphocyte classes or subsets or a selective block in a specific metabolic pathway required only for the mitogenic response may be elucidated by further study.

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