IMMUNOLOGIC PROPERTIES OF BACTERIAL LIPOPOLYSACCHARIDE (LPS)

III. Genetic Linkage between the In Vitro Mitogenic and In Vivo Adjuvant Properties of LPS*

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Bacterial lipopolysaccharide (LPS)[1] has among its broad spectrum of immunologic activities the capacity to modulate the induction of a specific state of tolerance in mice to the thymus-dependent antigen human IgG (HGG), into a specific state of immunity to HGG (1). Mice treated with LPS shortly after the injection of a tolerogenic dose of deaggregated HGG (DHGG) not only fail to become tolerant to HGG (2), but demonstrate a delayed primary response to HGG, and also respond anamnestically to a subsequent immunogenic challenge of aggregated HGG (AHGG) (1). This phenomenon, which has been viewed as a very stringent test of an adjuvant effect (3), was originally described by Claman (4) with bovine gamma globulin tolerance in mice, and has also been seen more recently by Ornellas et al. (5) with sheep gamma globulin tolerance in rats.

Recent studies have been directed toward precisely defining the cellular basis of this modulatory effect of LPS on HGG tolerance. It was found that the immune response to HGG which is seen as the result of dual treatment of mice with DHGG and LPS appears to occur in spite of the normal induction of tolerance in both HGG-specific thymocytes (1) and peripheral T cells (6). These observations suggest that LPS not only prevents tolerance induction in B cells, but also overcomes the normal requirement for HGG-specific T-helper cells. Furthermore, a positive correlation exists between the capacity of LPS to

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[1] *Abreviations used in this paper:* AHGG, aggregated HGG; DHGG, deaggregated HGG; HGG, human IgG; LPS, bacterial lipopolysaccharide; PFC, plaque-forming cells.

modulate HGG tolerance in vivo and its ability to induce B-cell mitogenesis in vitro. One of the most striking correlations between these two properties of LPS is the observation that LPS fails to inhibit tolerance induction to HGG in the C3H/HeJ mouse (7), a unique strain whose B cells are refractory to LPS-induced mitogenesis (7-10). The lack of an adjuvant effect of LPS in the C3H/HeJ is not due to the inability of this strain to respond to the antigen HGG, since it responds as well as other strains to an immunogenic challenge with AHGG (7). This correlation, taken together with the observation that HGG-specific T cells are tolerant in animals responding to the combination of DHGG and LPS, led to the concept that interference with tolerance induction by LPS results from a direct mitogenic effect of LPS on HGG-specific B cells (1, 7). As in the case of its mitogenic activity (11-16), the adjuvant effect of LPS may also occur without the participation of either T cells or macrophages.

This view is supported by the studies reported here which analyze the genetic control of the adjuvant and mitogenic properties of LPS. The refractory state of B cells from the C3H/HeJ to LPS-induced mitogenesis is a condition previously reported to be due to a single autosomal dominant gene which is not linked to either *H-2* or heavy chain allotype loci (9). Results of a backcross linkage analysis suggest that this gene may be identical to the one which limits the in vivo adjuvant activity of LPS in the C3H/HeJ strain.

Materials and Methods

Mice. C3H/HeJ (*H-2*^{k}) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The breeding nucleus of the C3H.SW-Ig-1^b (CWB/13) strain was obtained from Dr. Leonard Herzenberg, Stanford University, Stanford, Calif. C3H.SW-Ig-1^b (*H-2*^{b}) differs in IgG2a immunoglobulin allotype from other C3H strains, which are Ig-1^a. (C3H/HeJ × CWB) F_1 and (F_1 × C3H/HeJ) backcross mice were bred at the Salk Institute, San Diego, Calif.

Antigens. HGG (provided by the American Red Cross National Fractionation Center with the partial support of National Institutes of Health grant 13881[HEM]) was prepared either as the immunogen, AHGG, or as the tolerogen, DHGG, by methods described previously (17).

Lipopolysaccharide. Escherichia coli K235 LPS, prepared by a phenol-H_2O extraction procedure (18), was generously supplied by Abbott Laboratories, North Chicago, Ill., through Dr. Floyd C. McIntire, School of Dentistry, University of Colorado Medical Center, Denver, Colo.

Allotype Determination. Backcross mice were tested for the presence of the Ig-1^b allotype of the CWB grandparent by using Ouchterlony double-diffusion testing with a BALB/c anti-BAB/14 (Ig-1^a anti-Ig-1^b) antiallotype serum (9).

H-2 Typing. The backcross mice were tested for the presence of the *H-2*^{b} allele from the CWB grandparent by using a modification (9) of the polyvinylpyrrolidone technique (19) with a congenic *H-2*^{k} anti-*H-2*^{b} typing serum prepared as previously described (9).

Mitogenic Responses to LPS. Spleen cell suspensions were cultured and assayed for mitogenic responsiveness to LPS by uptake of [^{3}H]thymidine as detailed elsewhere (9).

Hemolytic Plaque Assay. Antibody-forming cells specific to HGG were enumerated using the Jerne plaque assay (20) in which HGG was covalently coupled to goat erythrocytes (Colorado Serum Co., Denver, Colo.) (21). Indirect plaque-forming cells (PFC) were developed with rabbit antimouse γ-globulin used at a concentration previously determined to be optimal in the assay.

Results

In order to determine whether the in vitro mitogenic and in vivo adjuvant properties of LPS are controlled by the same gene, a backcross linkage analysis was performed utilizing mice that were the progeny of a cross between the responder (C3H/HeJ [*H-2*^{k}, Ig-1^a] × CWB [*H-2*^{b}, Ig-1^b]) F_1 and the nonresponder

C3H/HeJ parent. These backcross progeny were subsequently tested individually for their ability to support mitogenic and adjuvant responses to LPS. In addition, two other genetic markers were also assessed which have previously been shown to be involved in controlling immunocompetence in mice, namely, *H-2* (22) and immunoglobulin heavy-chain allotype (23-25) loci. All mice were prebled for determination of the $H\text{-}2^b$ type and $Ig\text{-}1^b$ allotype characteristic of the responder grandparent strain, and 10 days later these mice were injected intraperitoneally with 2.5 mg of DHGG and 3 h later with 25 μg of *E. coli* K235 LPS. On day 20, animals were then challenged intravenously with 400 μg of AHGG, and 5 days later, the spleens were removed and single cell suspensions prepared. Separate aliquots from individual spleens were then: (*a*) incubated with LPS in vitro in order to assess their mitogenic responsiveness to LPS, and (*b*) assayed for their content of indirect PFC specific to HGG. Two groups of animals, totaling 24 mice, were tested in this manner. The results shown in Table I indicate clearly that LPS interfered with the induction of tolerance to HGG, as evidenced by the vigorous PFC responses to AHGG, but only in those mice which were genetically capable of supporting an in vitro mitogenic response to LPS. The remaining animals (9 out of 24) which were genetic nonresponders to LPS as a mitogen were also nonresponders to LPS as an adjuvant.

The fact that these two properties of LPS segregate together strongly suggests that they are controlled by the same gene. In contrast, these properties segregated independently from the expression of either *H-2* or heavy-chain allotype loci, and moreover, were not sex linked (Table I). This finding is consistent with earlier work demonstrating that mitogenic responsiveness to LPS in mice is a condition that is not genetically linked to any of these loci (9).

Control experiments utilizing additional groups of animals established that all backcross mice tested could be readily tolerized when treated with DHGG alone, or were equally responsive when immunized with AHGG alone (data not shown). Thus, as reported previously (7), adjuvanticity is directly attributable to the genetic capability of mice to respond mitogenically to LPS, rather than to recognize either the tolerogen or immunogen.

Discussion

The present studies represent the synthesis of two avenues of research undertaken independently in our laboratories that have been directed toward understanding the basis of two immunologic activities of LPS: on the one hand, the definition of the cellular basis of the adjuvant effect of LPS in vivo, and, on the other hand, the genetic basis of the mitogenic effect of LPS in vitro. The genetic analysis reported here and elsewhere (9) suggests that the expression of a single, autosomal dominant gene controls the capacity of C3H/HeJ mice to respond to both the mitogenic and adjuvant activities induced by LPS. The importance of this observation lies in the fact that it provides the strongest evidence of a positive correlation between these two LPS properties, since previous experiments had revealed this relationship by less direct means. Thus, experiments relating the immunologic effects of LPS to its chemical structure demonstrated that both its mitogenic and adjuvant activities are properties that could be attributed to the same distinct structural region of the LPS molecule, namely

TABLE I

*Genetic Linkage between the Mitogenic and Adjuvant Properties of LPS**

Mice	Sex	Ig-1	*H-2*	Mitogenic response, CPM/culture (E-C)	HGG response, indirect PFC/10^6 spleen cells
Group 1					
4	♀	—	—	6,200	520
5	♀	b	*b*	8,790	540
6	♀	—	—	10,390	696
7	♀	b	*b*	12,510	1,298
8	♀	b	*b*	40	0
11	♂	—	—	10,150	65
12	♂	—	—	23,500	405
13	♂	—	—	880	0
14	♂	b	*b*	25,380	410
16	♂	—	—	30,140	247
17	♂	b	*b*	0	1
18	♂	—	*b*	13,460	1,000
Group 2					
1	♂	—	*b*	8,240	544
2	♂	b	*b*	280	2
3	♀	b	*b*	570	0
4	♀	—	—	0	0
5	♀	—	—	16,130	264
10	♀	—	—	8,660	756
15	♀	—	*b*	0	0
16	♀	b	*b*	0	0
17	♀	—	*b*	14,880	274
18	♀	—	*b*	22,890	435
19	♂	—	—	0	0
21	♀	b	*b*	24,620	506

* (C3H/HeJ × CWB) F_1 mice were backcrossed to C3H/HeJ mice and the progeny were initially prebled for determination of Ig-1^b allotype and *H-*2^b type of the responder grandparent. *Ig-1* is the allotype locus for IgG2a immunoglobulin. CWB is Ig-1^b, C3H is Ig-1^a; thus, phenotype b = genotype b/a and — = a/a. CWB is *H-*2^b, C3H is *H-*2^k; thus, phenotype b = genotype b/k and — = k/k. 10 days after prebleeding, these mice were injected intraperitoneally with 2.5 mg of DHGG and 3 h later intravenously (i.v.) with 25 μg of *E. coli* K235 LPS. 20 days after this treatment, mice were challenged i.v. with 400 μg of AHGG, and 5 days later spleen cells were harvested and aliquots from individual mice were assayed for indirect PFC specific to HGG, and tested for mitogenic responsiveness to LPS. Mitogenic responses were assayed after 66 h of incubation of cells with 10 μg/ml of *E. coli* K235 LPS by a 6 h pulse with [^{3}H]thymidine. Results are expressed as the arithmetic mean of triplicate cultures after subtraction of background into saline-treated controls (E-C).

lipid A (13). Similarly, studies of the effects of chemical alteration of LPS on its immunologic activities have shown that base hydrolysis of LPS, a treatment that removes the ester-linked fatty acids from lipid A without affecting the structure of the O-antigenic polysaccharide, resulted in a loss of both its mitogenic and adjuvant effects (7). Lastly, a comparison of the immunologic activities of LPS in different mouse strains revealed that the C3H/HeJ mouse, unlike other mouse strains, was refractory to these two properties of LPS (7).

Because of these striking correlations, it is tempting to speculate that the

cellular basis of the adjuvant activity of LPS is restricted to B lymphocytes. The cellular location of the mitogenic defect in C3H/HeJ mice has been shown previously to reside exclusively at the level of B lymphocytes, rather than at the level of accessory cells. This was demonstrated by the fact that in vitro admixture of spleen cells obtained from responder and nonresponder mice resulted in neither enhancement nor suppression of the mitogenic response to LPS (reference 26 and footnote 2). Since the same gene appears to limit both the mitogenic and adjuvant properties of LPS in the C3H/HeJ, an intrinsic defect in B cells is most likely also responsible for the lack of an adjuvant effect of LPS in this strain, a conclusion which supports the concept that accessory cells are probably not involved in the adjuvant effect of LPS reported here.

Results obtained from other experimental systems suggest that the enhancing effect of LPS on antibody responses may be totally dependent on antigen-specific T cells, either because LPS has a direct potentiating effect on T cells, or an indirect effect on these cells via LPS-activated macrophages (27-29). It is difficult to completely rule out the possibility of such accessory cell involvement in the present experiments. For example, it is likely that the single gene that controls LPS-induced mitogenic responses in B cells is also expressed in other cell types, such as T cells and macrophages. Although the presumptive expression of this gene in these accessory cells appears to exert no significant influence on the mitogenic activity of LPS on B cells, it may have a profound influence on the adjuvant property of LPS. It is also possible that the gene expressed in B cells which regulates the mitogenic response to LPS is merely linked closely to a different gene, expressed in accessory cells, which limits the adjuvant response. In this case, analysis of a larger number of mice may reveal recombinants between these two LPS properties. Nonetheless, it should be stressed that a normal complement of antigen-specific T cells is not necessary for the effect of LPS on HGG tolerance, since LPS appears to have no influence on the induction of tolerance to HGG in specific T cells (1, 6).

The coordinate expression of the mitogenic and adjuvant properties of LPS not only suggests that the cellular site of action of LPS as an adjuvant is confined to B lymphocytes, but also suggests that its subcellular mode of action may result from the delivery of a signal to antigen-specific B cells which is a stimulus for mitogenesis. This interpretation would appear to be most compatible with the two signal model of immunity proposed by Bretscher (30) and Watson et al. (31). Within this conceptual framework, the role of a mitogenic signal would be to divert B cells from the tolerogenic pathway initiated by the interaction of DHGG with Ig receptors clonally expressed on specific B cells (signal 1), to the inductive pathway as a consequence of the interaction of the lipid A moiety of LPS with a LPS "receptor" non-clonally expressed on all B cells (signal 2), perhaps analogous to that found on human erythrocytes (32). Whether the gene which limits responsiveness to LPS does so by controlling the interaction of LPS with this receptor, or by regulating an LPS-specific event subsequent to this interaction,

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[2] Skidmore, B. J., J. M. Chiller, and W. O. Weigle. 1975. Immunologic properties of bacterial lipopolysaccharide (LPS). IV. Cellular basis of the unresponsiveness of C3H/HeJ mouse spleen cells to LPS-induced mitogenesis. Manuscript in preparation.

is at present unknown. If the gene defect in C3H/HeJ mice does control the expression of LPS receptors, it apparently does not quantitatively reduce the capacity of cells to bind LPS (26).

An alternative to the two-signal mechanism has recently been proposed by Coutinho and Möller (33, 34), who suggest that the only signal required to activate B cells is a single mitogenic stimulus provided either by activated T cells or by the antigen itself, in the case of thymus-dependent and thymus-independent antigens, respectively. Mitogenic stimulation by LPS, however, appears not to be sufficient to elicit an HGG response. That is, there is an absolute requirement for both DHGG and LPS in this system (1). This synergy can be explained within the context of the one signal model only if DHGG is required not for the delivery of a specific antigenic signal, but to facilitate in some fashion the delivery of a nonspecific mitogenic signal. This may occur, for example, by recruitment of LPS-activated macrophages through the interaction of the Fc region of HGG with the Fc receptor on macrophages (35), which may in turn secrete mitogenic factors (36). Such a mechanism must be considered in view of the inductive role ascribed to the macrophage (37).

It is clear that to reach a firm conclusion will necessitate a totally in vitro analysis of both tolerance and immunity in this system, so that the individual contributions of antigen, LPS, and various cell types can be better assessed. The LPS-specific defect in the C3H/HeJ mouse strain should provide a valuable tool for such an analysis.

Summary

The mechanism was investigated underlying the activity of bacterial lipopolysaccharide (LPS) as an adjuvant of antibody formation as assessed by its capacity to modulate the induction of tolerance in mice to the antigen human IgG (HGG) into a state of immunity to HGG. The adjuvant activity of LPS was found to be closely correlated with its ability to function as a B-cell mitogen. This correlation was revealed by an analysis of the genetic control of the mitogenic and adjuvant properties of LPS utilizing the refractory state inherent in the C3H/HeJ mouse strain to these activities of LPS. Thus, mice that were the progeny of a backcross between the nonresponder C3H/HeJ parent and the responder (C3H/HeJ × CWB) F_1 hybrid were individually typed for responsiveness to LPS, as an adjuvant and as a B-cell mitogen. It was found that LPS interfered with tolerance induction to HGG in vivo only in those backcross progeny whose spleen cells were also capable of responding mitogenically to LPS in vitro, demonstrating that the adjuvant and B-cell mitogenic properties of LPS are genetically linked. In contrast, these properties were observed to segregate independently from either *H-2* or heavy chain allotype loci, and were not sex linked. These results are compatible with the concepts that, in this system, (*a*) the cellular site of action of LPS as an adjuvant is confined to B cells, and (*b*) the subcellular mode of action of LPS as an adjuvant may involve the delivery of a "signal" to B cells which is a stimulus for mitogenesis.

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