Brief Definitive Report

SUPPRESSION OF THE IMMUNE RESPONSE IN C3H/HeJ MICE BY PROTEIN-FREE LIPOPOLYSACCHARIDES*

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In studies to date, the C3H/HeJ strain of mice has been shown to be unresponsive to all the investigated aspects of endotoxicity exerted by phenol extracted endotoxic lipopolysaccharides (LPS), e.g., adjuvant action (1, 2), mitogenicity (3–5), polyclonal B lymphocyte Ig synthesis (1, 6), endotoxic shock (5), enhancement of nonspecific resistance to infection (7), leukocyte changes (8), protection against X-irradiation (9), negation of tolerance induction (1, 10), and inducement of glucocorticoid antagonizing factor (11). Watson and Riblet have published evidence that this unresponsiveness of the C3H/HeJ mouse is determined by a single autosomal, dominant gene which is not linked to the H_2 or heavy chain allotype loci (4).

On the other hand, several endotoxic functions have been shown to be elicited in the C3H/HeJ mouse by LPS extracted with butanol or trichloroacetic acid (Boivin type) (1, 12). This product has been shown to contain a low molecular weight protein contaminant which probably is responsible for the observed stimulation of mitogenicity, polyclonal B-cell activation, and inhibition of tolerance induction (13, 14). Despite the presence of this protein, however, the butanol extracted LPS was incapable of adjuvant action in this mouse strain.

In contrast to the adjuvant action of bacterial endotoxins, which occurs when LPS is given with antigen, an opposite, suppressive effect was noted in rabbits when LPS was introduced before the antigen (15). Franzl and McMaster, in their thorough study of this effect in mice, noted that the dose-dependent inhibition of hemolysin production was paralleled by a ⅔ decrease in the normal weight of the thymus when LPS was given 1–2 days before antigen (16, 17). Also negated was the early and excessive proliferation of large pyroninphilic cells in the white pulp of the spleen, cortical regions of lymph nodes, and activated germinal centers that is characteristic of mice injected with endotoxin and antigen together (18). Recent renewed interest in this phenomenon has resulted in an oscillating effect with time of injection of LPS relative to antigen in ICR mice being noted (19). In addition, Persson has reported evidence that LPS suppression in the A/Sn/C57Bl F_1 hybrid was due to the generation of nonspecific, B-suppressor cells (20), while others have shown that the secondary response of several mouse strains could be suppressed if the LPS was given either before (21) or after (3) antigen. The latter authors reported, however, that the LPS did not suppress antibody-forming cells in the C3H/HeJ mouse when 1 μg was given 1 day after antigen.

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To extend our characterization of the unresponsiveness of the C3H/HeJ mouse to the action of LPS, we tested the capacity of a protein-free and a Boivin type endotoxin to induce suppression of the primary immune response in this unique mouse strain. Surprisingly, suppression of plaque-forming cells (PFC) formation was readily induced with both preparations and found to be transferrable with spleen cells of LPS treated C3H/HeJ mice. To our knowledge, this is the first report of an effect by a protein-free LPS on the immune response in the heretofore unresponsive C3H/HeJ mouse.

Materials and Methods

Mice. The C3H/HeJ mice and their responsive genetic counterpart, the C3H/HeB/FeJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. After establishing the endotoxin induced suppression, these mice were bred in our own laboratory by brother-sister matings. The mice were utilized at 2- to 9-mo of age.

Antigen. Sheep erythrocytes (SRBC) were obtained from Colorado Serum Co., Denver, Colo. as sterile lamb's blood in Alsever's solution. The cells were washed three times with Moller's modified salt solution before use. 5×10^8 cells were injected intraperitoneally; in the in vitro experiments, 10^7 SRBC were added to each culture dish.

Suppressive Agents. Lipopolysaccharide was prepared by the Boivin procedure from *Serratia marcescens*, and the protein-free, purified glycolipid from *Salmonella minnesota*, R 595. Immunochemical characterization of the former (15) and the latter (22) has been published previously. 20 μg of the Boivin preparation and 50 μg of the glycolipid after sonication were injected intraperitoneally, while 0.02 μg of the former or 0.05 μg of the latter were added to in vitro culture dishes. Polyadenylic acid, lot 81, and polyuridylic acid, lot 92, were purchased as the potassium and ammonium salts, respectively, from Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind. Equal amounts of the individual homoribopolymers were combined in 0.15 M NaCl at room temperature for 1 h; 200 μg of the complex was injected i.p. into each mouse. Injection of each agent was performed on day −2 or 0 in relationship to antigen administration.

Measurement of Suppression. Spleen cells were removed from C3H/HeJ mice sacrificed by cervical dislocation and single cell suspensions were prepared on day 0 for the in vitro tests and on day 4 for the in vivo experiments. Cells removed on day 0 were cultured according to the technique of Mishell and Dutton (23). They were suspended to a concentration of 10^7 cells/ml and dispensed in 1-ml portions into 35 × 10-mm Falcon plastic culture dishes. The transfer experiments utilized 0.5 ml (5×10^6) of normal cells from uninjected mice mixed with 0.5 ml (5×10^6) of cells from mice previously treated with endotoxin on day −2. 5×10^6 SRBC were then added to each culture dish on day 0, and the culture plates were incubated for 4 days in a gas tight humidity chamber containing 7% O_2, 10% CO_2, and 83% N_2. They were rocked at 7 cycles/min at 37°C and fed daily with a pH balanced nutritional supplement. The PFC from both the in vitro and in vivo studies were assayed using the localized hemolysis in gel technique as modified by Coutinho et al. (24).

Results

The effect of injection into C3H/HeJ mice of a Boivin type endotoxin and a purified glycolipid endotoxin two days before antigen is compared in Fig. 1 with these products added with antigen. It may be seen that, as expected, neither the Boivin type LPS or the glycolipid acted as an adjuvant in this strain when introduced together with antigen. Each of these lots of LPS previously had been shown to act as an adjuvant in numerous other strains of mice. Poly A:U, a known non-LPS adjuvant, served as the positive reference control, and documented the selective nature of the deficiency to LPS in this strain. However, when either LPS was injected 2 days before antigen, a profound inhibition averaging 75% or better was observed. Poly A:U, whose immunorepertoire also includes suppression of PFC when injected before

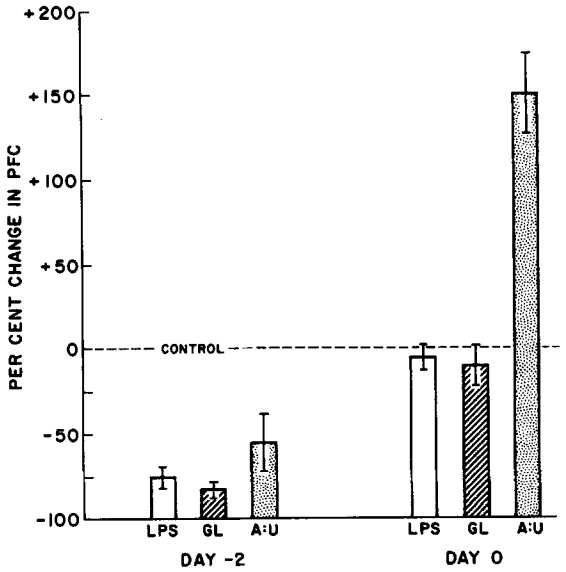

FIG. 1. Inhibition of PFC formation by i.p. injection of 20 μg Boivin type LPS or 50 μg glycolipid 2 days before i.p. injection of 5×10^8 SRBC. A:U = 200 g polyadenylic-polyuridylic acid complex injected i.p. on days indicated. Mice sacrificed and PFC determined 4 days after antigen. Mean number of PFC in control spleens = 1,630 ± 273. Values are ± standard deviation.

TABLE I

Suppression of PFC Response of Normal C3H/HeJ Spleen Cells by Endotoxin Exposed C3H/HeJ Spleen Cells

Exposure to agent	Spleen cells in culture	Suppression of PFC*
		%
Day −2‡	LPS Treated	65 ± 13
	LPS Treated + normal (1:1)	51 ± 12
	Glycolipid treated	49 ± 8
	Glycolipid treated + normal (1:1)	44 ± 17
Day 0§	LPS Treated	0 ± 0
	Glycolipid treated	3 ± 4

* Average of three experiments ± standard deviation.

‡ 20 μg LPS or 50 μg glycolipid injected i.p. 2 days before removal and culture of 10^7 total spleen cells with 10^7 SRBC/culture dish.

§ 0.02 μg LPS or 0.05 μg glycolipid plus 10^7 SRBC added in vitro to 10^7 cultured spleen cells.

antigen (25), again served as a positive reference control. Thus, although LPS failed to function as an adjuvant in C3H/HeJ mouse in accordance with what has been reported previously (1, 2), both preparations were capable of inducing the opposite action, suppression.

To determine whether such suppression might be transferrable via spleen cells and dampen antibody synthesis by normal spleen cells, coculture experiments were performed with a 1:1 mixture of spleen cells from mice previously injected with LPS, and normal cells, cultured with antigen. As may be seen in Table I, addition of antigen in vitro to cultured spleen cells removed from C3H/HeJ mice injected with either glycolipid or Boivin type LPS 2 days earlier, resulted in a 49–65% inhibition of PFC formation. Coculture of 5×10^6 of such inhibited cells with an equal number of

normal spleen cells from uninjected C3H/HeJ mice resulted in transfer of this suppressive property, diminishing the normal PFC capability by a similar order of magnitude, 44–51%. In contrast, coculture of spleen cells from mice receiving LPS on day 0 did not result in any inhibition of the normal cell response. Parallel control studies in C3H/HeB/HeJ mice, which are susceptible to the action of LPS, showed the expected 56% inhibition when injected with LPS on day −2, and enhancement of the day 4 PFC when either LPS was introduced on day 0 together with antigen (data not shown).

Mitogenicity (stimulation index) of the glycolipid was minimal for spleen cells of the C3H/HeJ strain, but averaged approximately 20-fold in the C3H/HeB/FeJ strain. The Boivin type LPS was not significantly active in either of these mice. However, both the Boivin preparation and the glycolipid were strongly mitogenic for reference BALB/c spleen cells (23 and 22-fold, respectively).

Discussion

The above data show for the first time that the C3H/HeJ strain of mice is not refractory to all of the diverse biological activities of protein-free endotoxins derived from Gram negative bacteria. In previous studies, any biological action of LPS in this strain could be attributed to a protein contaminant. This is not the case with the inducement of suppressor action as illustrated in this manuscript, since Chen et al. (22) have analyzed this glycolipid preparation in detail and indicated it to be a protein-free, chloroform-methanol soluble complex composed of lauric, myristic, palmitic, stearic, and beta-hydroxymyristic acids with only trace amino acids. Its biologic activities were comparable to the parent compound.

The greater implication of these data are, however, that the suppressor cell regulatory mechanism for the immune response induced by LPS is under a different genetic control than the helper function. In all studies to date, including this one, the C3H/HeJ mouse did not respond to the profound adjuvant action, as well as other properties (e.g., mitogenicity), of LPS. This indicates that a gene product (LPS receptor?) is absent or diminished in this mouse strain. Verification is provided by the work of Melchers et al. (personal communication) who have shown that in the C3H/HeJ mouse only 1 in 10,000 B lymphocytes are reactive to LPS, as compared to a ratio of 1:3 in other strains. Consequently, a gene product(s) controlling suppression on the B cell may be different from that controlling other activities of LPS, or more likely, the LPS suppressive signal acts on a different target, the T cell or macrophage. Thus, we hypothesize that the feature of non-B-cell responsiveness to LPS still holds in this unique mouse strain with the induced suppressor cell being probably a T cell or macrophage. Current experiments are designed to test this hypothesis.

Summary

The experiments described herein demonstrate the plaque-forming cell response of C3H/HeJ mice can be suppressed by a Boivin type lipopolysaccharide (LPS) and a deproteinized glycolipid. Suppression was observed both in vivo and in vitro, and could be transferred to normal cells in coculture experiments. This newly discovered effect of LPS in C3H/HeJ mice indicates that the adjuvant and inhibitory action of LPS may be distinct phenomena which are under different genetic regulation. Thus,

the C3H/HeJ strain provides a convenient animal model for study of immunosuppression independent of the adjuvant effect.

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