Effect of Lipopolysaccharide (LPS) Injection on the Immune Responses of LPS-Sensitive Mice

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ABSTRACT. The effect of lipopolysaccharide (LPS) on humoral and cell-mediated immunity was assessed using LPS-sensitive C3H/HeN mice. A single injection of LPS significantly decreased the anti-sheep red blood cells (SRBC) antibody titers, but not the number of anti-SRBC antibody producing spleen cells. In contrast, double LPS injection did not significantly decrease the anti-SRBC titers and even increased the number of anti-SRBC antibody producing spleen cells. Similarly, single LPS injection significantly suppressed the swelling of the footpad, but double LPS injection caused milder suppression. These results suggest that a difference in the level and timing of exposure to LPS may influence the immune response to infection or vaccination.

KEY WORDS: C3H/HeN, immune response, lipopolysaccharide, tolerance.

In an effort to improve the health of domestic animals, chronic infection is recently being paid more attention than acute epidemic infection, as most acute infections are under control. Since many chronic infections are caused by gram-negative bacteria, vaccines against these microbes have been increasingly produced, most of which are inactivated whole-cell vaccines. Lipopolysaccharide (LPS), which is a common component of the cell walls of gram-negative bacteria, is capable of eliciting a wide variety of pathophysiological effects such as endotoxin shock, tissue injury, and lethality in both man and animals [8, 10]. It has been reported that LPS can induce apoptosis of lymphocytes and the elevation of serum level of tumor necrosis factor (TNF)-α in both piglets and mice [4, 5, 13, 14]. Moreover, it has been recently hypothesized that Toll-like receptor 4 (TLR4) is the critical receptor for LPS signaling in host defense, and the innate immune system may be involved in the regulation of the adaptive immunity, which is mediated by B and T lymphocytes [16, 21]. However, little is known about the effects of LPS on the immune response of domestic animals as well as the mechanism behind.

In this report, we describe the effect of single and double LPS injections on the immune responses of an LPS-sensitive mouse strain as a model for domestic animals.

MATERIALS AND METHODS

LPS: LPS from Escherichia coli O55:B5 extracted by the hot phenol-water extraction method was purchased from Difco laboratories, U.S.A. and was suspended in pyrogen-free saline (Otsuka, Tokyo, Japan).

Quantification of antibody titers and antibody producing spleen cells: Eight-week-old female mice of the LPS-sensitive C3H/HeN strain (Japan SLC, Inc., Shizuoka, Japan) were intravenously immunized with 0.2 ml of 5% of sheep red blood cells (SRBC) and boosted 14 days later in an identical manner. The single LPS injection (single LPS) group was intravenously injected with 1 mg/kg of LPS 2 days before the booster immunization (n=5). Accordingly, the double LPS injection (double LPS) group was intravenously injected 2 days before both the priming and boosting immunizations (n=5). As a positive control, 5 mice were immunized with 5% of SRBC twice in the same way without LPS injection. Negative controls were injected with pyrogen-free saline. The anti-SRBC antibody titers and the number of anti-SRBC antibody producing spleen cells were measured 7 days after the second immunization by the SRBC hemagglutination test and by use of a chamber made up of two glass slides [2], respectively.

Delayed type hypersensitivity assay: LPS-sensitive C3H/HeN mice were intravenously immunized with 0.2 ml of 0.01% of SRBC. Three days after immunization, mice were challenged by injection of 0.03 ml of 20% SRBC intradermally into the left footpad. Delayed type hypersensitivity (DTH) responses were determined 3 days after challenge as the percentage increase in thickness of the left footpad compared with the right footpad, which was injected with 0.03 ml of pyrogen-free saline. The single LPS group was intravenously injected with 1 mg/kg of LPS 3 days before the immunization (n=10) and the double LPS group was injected both 3 days and 17 days before the immunization (n=6). Positive controls were treated in the same way with-
out LPS injection (n=6).

**Flowcytometry analysis:** C3H/HeN mice were injected with 1 mg/kg of LPS at day 0 (single LPS) or day 0 and 14 (double LPS). Either 3 or 6 animals were processed for flowcytometry at each time point: 0, 2, 5, 14 days post injection (dpi) and 2 days after the second LPS injection (14+2 dpi). For flowcytometry analysis, single cell suspensions containing approximately 10^6 cells were stained with monoclonal antibodies for 20 min at 4°C after incubation with anti-mouse Fcγ II/III receptor antibody (2.4G2) (PharMingen, San Diego, CA, U.S.A.) for 20 min at 4°C. Monoclonal antibodies used for staining included: fluorescent isothiocyanate-conjugated (FITC)-anti mouse CD3 epsilon (PharMingen), FITC-anti mouse CD8a (Ly-2) (PharMingen), Phycoerythrin-conjugated (PE)-anti mouse B220 (PharMingen), PE-anti mouse CD4 (L3T4) (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Analysis was conducted on a FACScan (Becton Dickinson) and the data were analyzed by software Consort 30 (Becton Dickinson). Dead cells and debris were gated out of the analysis on the basis of forward and side light scatter. The flowcytometry data were presented as the number of cells by calculating from the number of total cells of the organ and the percentage of each cell population.

**Statistic analysis:** Differences between groups were determined by analysis of variance using the StatView J-4.5 (Abacus Concepts, Inc., Berkeley, CA, U.S.A.).

**RESULTS**

**The anti-SRBC antibody titers and the number of anti-SRBC antibody producing spleen cells:** When LPS was injected 2 days before the second immunization (single LPS), the anti-SRBC antibody titers were significantly lower than those of the positive controls (p< 0.01). However, when mice were pretreated with the same dose of LPS 14 days before the first LPS injection (double LPS), the antibody titers were significantly higher than those of mice given a single LPS injection (Fig. 1).

When LPS was injected 2 days before the second immunization (single LPS), the number of anti-SRBC antibody producing spleen cells were similar to those of the positive control group. However, when mice were pretreated with the same dose of LPS 14 days before the first LPS injection (double LPS), the increase in the number of these cells was significantly higher than those of mice given a single LPS injection (Fig. 2).

**Influence of LPS injection on delayed type hypersensitivity responses:** In the group given a single LPS injection, the footpad swelling was significantly lower than that of the positive control group (p< 0.01). However, swelling was significantly higher in the double LPS group than the single LPS group (p< 0.05) (Fig. 3).

**Changes of thymus weight and leukocyte subpopulation in thymus and spleen by the LPS injection:** In order to analyze the effect of LPS on immune system further, we also examined the thymuses and spleens of the mice treated with either single or double LPS injection by flowcytometry and histology.

The weight of the thymus decreased at 2 dpi, accompanied by cortical atrophy by histological observation, and recovered to the negative control level by 14 dpi (Fig. 4a). Flowcytometry showed that the number of CD4+CD8+ cells significantly decreased at 2 dpi, almost disappeared at 5 dpi and recovered by 14 dpi (Fig. 4b), coinciding with the observation in the weight and histology. In contrast, 2 days after the second LPS injection, neither the weight nor the number of CD4+CD8+ cells in thymus decreased to the same level as 2 days after the first LPS injection i.e. the suppressive effect of LPS was reduced after double LPS injection.

In the spleen, B cells represented by B220+ cells did not change significantly in number after the first LPS injection,
but significantly increased after the second injection (Fig. 4c).

DISCUSSION

In this study we have shown that a single LPS injection into SRBC-immunized mice induces suppression of anti-SRBC antibody production and footpad swelling. Double LPS injection abrogated this immunosuppressive effect and enhanced the number of anti-SRBC antibody producing spleen cells.

T lymphocyte may be mainly classified into CD8+ cytotoxic T cell (CTL) which participates in the cell-mediated immune response and CD4+ helper T (Th) cell which may be divided into two functionally different Th1 and Th2 subset [11]. The Th1 cells are crucial for the activation of the CTL, whereas Th2 cells provide help to B cells leading to the production of antibodies [19, 20]. We revealed that the single LPS injection dramatically induced the depletion of CD4+CD8+ cells in the thymus at 2 dpi and completely recovered by 14 dpi (Fig. 4b). Moreover, the number of both CD4+ CD8- and CD4-CD8+ cells in the thymus decreased approximately one third of the control level at 5 dpi (data not shown). These results, therefore, suggest that the single LPS injection suppress the humoral and cell-mediated immune responses.

In our result, the change of B lymphocyte in the spleen corresponds to the change in number of anti-SRBC antibody producing spleen cells (Fig. 2 and Fig. 4c). T cells represented by CD3+ cells decreased after the first LPS injection in the spleen and almost recovered by 14 dpi, whilst they did not decrease after the second LPS injection (data not shown). Antibody production depends on B cell activation by antigen followed by the stimulation by antigen-specific Th cells [20]. Thus, the change in the number of B and T
cells may partly explain the observation that the anti-SRBC antibody titers were significantly lower following single LPS injection, but not in double LPS injection. It is also suggested that the function of T cells did not recover enough to activate antibody production by B cells in the double LPS injection, in which the anti-SRBC antibody titers did not increase compared with the positive control group.

It is well known that LPS can directly activate polyclonal B cells to differentiate into antibody producing cells [1]. However, this in turn may transiently exhaust the existing memory B cells that can interact with Th cells to stimulate proliferation, resulting in a suppressive effect on antibody production. This may partly explain the results presented herein that show a single LPS injection induces significant suppression of antibody titers, but does not affect on the number of antibody producing spleen cells. Simultaneous injection of SRBC and lipid A from Salmonella Minnesota R595 enhances the number of IgM-plaque-forming spleen cells in mice 3 and 4 days after injection and the serum IgM titer 4 and 5 days after injection [7]. Thus, the timing of LPS injection may complicate the consequent immune response to particular antigens.

It has been reported that LPS isolated from different bacterial strains exhibits a wide diversity in strength of adjuvant action in the induction of delayed type hypersensitivity to ovalbumin measured by footpad reaction in mice when antigen is simultaneously injected with each LPS [15]. However, it has also been reported that LPS can induce apoptosis mainly of CD4+CD8+ thymocytes and destruction of T cell area in the spleen in mice 1 to 3 days after LPS injection [4, 5, 14, 23]. Our results confirmed the latter observation, which may have caused the suppression of cell-mediated immunity measured by footpad reaction when mice were treated with single LPS injection 3 days before immunization.

Compared with the single LPS injection, the double LPS injection induced much less suppressive effects on both humoral and cell-mediated immune responses. In addition, we also observed that single LPS injection induced significant levels of mRNA expression of TNF-α and interferon-gamma in thymus 1 hr after injection, whereas in double LPS injection, the levels of these cytokines were dramatically reduced 1 hr after the second LPS injection (data not shown). This phenomenon is described as a late phase tolerance when a second LPS injection 5 days or more after the first induces little biological response. Late phase tolerance is thought to be associated with the development of specific antibodies against the polysaccharide side chain of LPS [3, 22]. This may partly explain our results, however, the precise mechanism of late phase tolerance is yet to be understood. It has recently been revealed that induction of early phase tolerance was regulated by TLR4 expressed on antigen-presenting cells, such as macrophages and dendritic cells, and production of pro-inflammatory cytokines [12, 21]. It is assumed that late phase tolerance is also regulated by Th cells and pro-inflammatory cytokines produced by antigen-presenting cells. The further study into the mechanism of late phase tolerance should also be conducted.

Our data suggest that LPS may induce suppression in both humoral and cell-mediated immunity on LPS-sensitive animals. The results also suggest that a difference in the level and timing of exposure to LPS, which may mimic the differences in the conditions where animals are reared (e.g. specific pathogen free environment or natural environment) and/or the history of infection with gram-negative bacteria, may influence the outcome of immune response. This may have profound consequences on the immune responses elicited during infection or vaccination.

REFERENCES


