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# Inactivation of Suppressor T-Cell Activity by Nontoxic Monophosphoryl Lipid A

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**Treatment with nontoxic monophosphoryl lipid A (MPL), which was derived from a polysaccharide-deficient, heptoseless Re mutant of *Salmonella typhimurium*, was found to inactivate suppressor T-cell activity, as evidenced by a decrease in the degree of low-dose immunological paralysis expressed and an increase in the magnitude of the antibody response to type III pneumococcal polysaccharide. The effects produced, which could not be attributed to the polyclonal activation of immune B cells by MPL, were dependent upon the dose of MPL used, as well as the time when MPL was given relative to low-dose priming or immunization with type III pneumococcal polysaccharide. Neither amplifier nor helper T-cell activity was decreased by treatment with the same, or larger, doses of MPL. The significance of these findings to the use of MPL as an immunological adjuvant or an immunomodulating agent is discussed.**

Although bacterial lipopolysaccharides (LPSs) or endotoxins are considered to be potent immunopotentiating agents (14, 29; for a review, see reference 38), their use as immunological adjuvants is precluded by the fact that they are extremely toxic and pyrogenic for most animal species, even when given in small amounts (38). These effects have been attributed to the diphosphoryl lipid A portion of the LPS molecule (38, 39, 41); however, removal of a phosphate group from the reducing end of diphosphoryl lipid A yields monophosphoryl lipid A (MPL). MPL retains all of the beneficial properties of LPS and diphosphoryl lipid A (e. g., adjuvanticity and tumor necrosis activity), but is neither toxic nor pyrogenic in large doses (38–41, 46). This has renewed much interest in the use of MPL as an adjuvant as well as a therapeutic agent for the treatment of tumors (40).

Several factors might contribute, at least in part, to the ability of MPL to act as an immunological adjuvant. MPL (i) is mitogenic for bone marrow-derived precursors of antibody-forming cells or B cells, (ii) activates peritoneal macrophages, (iii) stimulates the production of interleukin 1, and (iv) promotes granulopoiesis through the release of endogenous colony-stimulating factor (41); however, the effect of treatment with MPL on thymus-derived (T) cells that regulate the magnitude of the antibody response has not yet been investigated in a systematic manner. In the present study, we examined the influence of treatment with MPL on the expression of suppressor, amplifier, and helper T-cell activity with reference to the antibody response to type III pneumococcal polysaccharide (SSS-III).

## MATERIALS AND METHODS

**Mice.** Female BALB/cByJ mice (age, 8 to 10 weeks; Jackson Laboratory, Bar Harbor, Maine) were used throughout this study. Female BALB/c athymic nude (*nu/nu*), as well as thymus-bearing (*nu/+*) littermate controls (age, 7 to 8 weeks), were obtained from the Frederick Cancer Research Center (Frederick, Md.).

**Antigens and immunization procedure.** The immunological properties of the preparation of SSS-III used and the method by which it was prepared have been described previously (1, 6, 7, 9–11). For immunization, mice were given a single intraperitoneal (i.p.) injection of an optimally immunogenic dose (0.5 μg) of SSS-III in 0.5 ml of saline. The magnitude of the antibody response produced was determined 5 days after immunization.

**Immunological methods.** The numbers of antibody-producing plaque-forming cells (PFCs) specific for SSS-III detected in individual mice provided a measure of the antibody response produced at the peak, i.e., 5 days after immunization with SSS-III. PFCs that made antibody of the immunoglobulin M (IgM) class (>90% of all PFCs found [1, 9]) were detected by a slide version of the technique of localized hemolysis-in-gel (13) by using indicator sheep erythrocytes coated with SSS-III by the $CrCl_3$ method (13). Polyethylene glycol 6000 (average molecular weight, 6,000 to 7,500; J. T. Baker Chemical Co., Phillipsburg, N.J.) was added to the reaction mixture (melted agarose) at a final concentration of 0.25% (wt/vol) to improve the quality of the plaques found. Corrections were made (by subtraction) for the number of background sheep erythrocyte-specific PFCs present, so that only values for PFCs that made antibody specific for SSS-III are considered. The values obtained (PFC per spleen), which are log normally distributed (25), are expressed as the geometric mean (antilog) of the $\log_{10}$ number of PFCs per spleen for groups of similarly treated mice. This provides a reasonably good measure of the magnitude of the total antibody response produced, since SSS-III-specific PFCs are detected only in the spleens of immunized mice (1, 6, 30).

Student's *t* test was used to assess the significance of the differences observed. Differences were considered to be significant when probability (*P*) values of $<0.05$ were obtained.

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**Assessment of suppressor, amplifier, and helper T-cell activity.** The degree of unresponsiveness induced 3 days after the administration (i.p.) of a single subimmunogenic dose (0.005 μg) of SSS-III provided a measure of the amount of suppressor T-cell activity present; such unresponsiveness (low-dose immunological paralysis) has been shown to persist for several weeks or months, to be antigen specific, and to be mediated by Lyt-$2^+$, L3T$4^-$ suppressor T cells (2, 33).

The degree of enhancement of the SSS-III-specific PFC response of mice treated with concanavalin A (ConA) was used to determine the relative amount of amplifier T-cell activity present. ConA (carbohydrate content, <0.1%) was purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.). It was dissolved in saline and given (i.p.) at a dose of 300 μg of ConA (in 0.2 ml of saline) 2 days after immunization with 0.5 μg of SSS-III. Such treatment favors the activation of amplifier T cells, as evidenced by the increased number of SSS-III-specific PFCs produced (4, 31).

The effect of helper T cells on the magnitude of the antibody response to SSS-III was evaluated by intravenous immunization with a helper T-cell-dependent form of this antigen, as described previously (32). This consists of SSS-III attached to an appropriate carrier (e.g., horse erythrocytes) to form a conjugate (SSS-III–horse erythrocytes). Pretreatment or priming with a subimmunogenic dose of carrier (horse erythrocytes) then permits mice to generate an antibody response upon immunization with amounts of the conjugate (SSS-III–horse erythrocytes) that would otherwise not be immunogenic in unprimed mice (32). Such an antibody response has been shown to be both specific for SSS-III and dependent upon the presence of carrier-specific helper T cells generated as a result of priming with carrier (15, 16).

**Assessment of polyclonal and mitogenic activities of MPL.** Cells secreting immunoglobulin of the IgM class were detected by a modification of the protein A plaque assay (26), in which indicator sheep erythrocytes were coated with protein A (Pharmacia), in the presence of 66 μg of $CrCl_2$ per ml as the coupling agent. The affinity-purified rabbit anti-mouse IgM used for the detection of non-antigen-specific IgM-secreting PFCs was provided by R. A. Asofsky of our laboratory. A dilution (50 μl in saline) known to reveal maximal numbers of IgM-secreting PFCs was added to the soft agarose reaction mixture before the addition of spleen cells. Results were expressed as $\log_{10}$ IgM-secreting PFCs per spleen ± standard error of the mean (SEM) for groups of mice immunized (i.p.) with 0.5 μg of SSS-III, with or without treatment with MPL.

To examine the mitogenicity of MPL, spleen cells from mice immunized (i.p.) with 0.5 μg of SSS-III were cultured in 96-well flat-bottom microtiter plates containing Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with glutamine (2 mM), essential and nonessential amino acids, 2-mercaptoethanol ($5 \times 10^{-5}$ M), sodium pyruvate (1 mM), D-glucose (4.5 mg/ml), and 10% endotoxin-free fetal bovine serum (lot 906026; Biofluids Inc., Rockville, Md.). Different numbers of spleen cells were cultured in the presence of different amounts of MPL (final volume, 200 μl) for 72 h at 37°C (5% $CO_2$ in air); during the last 6 h of culture, 1 μCi of [*methyl*-$^3$H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added. Then, the cells were harvested with a PHD cell harvester (Cambridge Technology, Watertown, Mass.) and collected on glass fiber filters. Radioactivity was detected with a scintillation counter (LS 8100; Beckman Instruments, Inc., Fullerton, Calif.).

**MPL.** MPL (average molecular weight, 1,718) was obtained from Ribi ImmunoChem Research, Inc. (Hamilton, Mont.). It was isolated from the polysaccharide-deficient, heptoseless Re mutant, *Salmonella typhimurium* G30/C21 as described previously (38). Lyophilized MPL was reconstituted to 1 mg/ml in distilled water containing 0.2% triethylamine. It was mixed thoroughly and sonicated briefly to obtain an opalescent stock solution which was stored at 4°C until use; the stock solution was diluted with saline to contain the desired amount of MPL to be administered (i.p.) in a volume of 0.2 ml of saline. Data on the toxicity and pyrogenicity of MPL, as well as of the parent LPS from which it was derived, have been published previously (38).

## RESULTS

**Effect of treatment with MPL on the expression and induction of low-dose paralysis to SSS-III.** Groups of mice were pretreated (primed) with a single injection (i.p.) of a subimmunogenic dose (0.005 μg) of SSS-III. Three days later, they were given (i.p.) an optimally immunogenic dose (0.5 μg) of SSS-III, with or without a single injection (i.p.) of MPL; and the magnitude of the SSS-III-specific PFC response was determined 5 days after immunization.

Priming with 0.005 μg of SSS-III resulted in a significant decrease in the magnitude of the PFC response to 0.5 μg of SSS-III, as expected (Table 1; groups A versus B, $P < 0.05$); such unresponsiveness (low-dose paralysis) has been shown to be antigen specific and mediated by suppressor T cells (2). The administration of 5 or 25 μg of MPL at the time of immunization with 0.5 μg of SSS-III did not alter the degree of low-dose paralysis expressed (Table 1; groups C and D versus group B, $P > 0.05$); however, low-dose paralysis was reduced in mice treated with 50 or 100 μg of MPL (Table 1; groups E and F versus group B, $P < 0.05$), although the resulting PFC response obtained was still less than that of unprimed immunized controls (Table 1; group A versus groups E and F, $P < 0.05$).

We also examined whether two injections of a relatively large dose (50 μg) of MPL would be more effective than one in reducing the degree of low-dose paralysis expressed. This appeared to be the case, although the results obtained greatly depended upon when MPL was administered relative to the day of immunization with 0.5 μg of SSS-III (Table 2). Two injections of 50 μg of MPL, given on the day before and the day of immunization with 0.5 μg of SSS-III (days −1 and 0, respectively), significantly reduced the degree of low-dose paralysis expressed (Table 2; groups B versus C, $P < 0.05$);

TABLE 1. Effect of treatment with different amounts of MPL on the expression of low-dose paralysis to SSS-III

| Group | MPL treatment (μg)[a] | SSS-III-specific PFC/spleen[b] |
|---|---|---|
| A | | 4.262 ± 0.074 (18,271) |
| B | | 3.435 ± 0.122 (2,723 ) |
| C | 5 | 3.509 ± 0.118 (3,228 ) |
| D | 25 | 3.641 ± 0.059 (4,377 ) |
| E | 50 | 3.842 ± 0.104 (6,942 ) |
| F | 100 | 3.994 ± 0.071 (9,866 ) |

[a] BALB/cByJ mice were given a single injection (i.p.) of 0.005 μg of SSS-III (except mice in group A) 3 days before immunization (i.p.) with 0.5 μg of SSS-III (all groups). MPL was given (i.p.) at the time of immunization with 0.5 μg of SSS-III.

[b] $\log_{10}$ SSS-III-specific PFC per spleen ± SEM for 8 mice 5 days after immunization (i.p.) with 0.5 μg of SSS-III; geometric means (antilogs) are given in parentheses.

TABLE 2. Effect of time of administration of MPL on the expression of low-dose paralysis to SSS-III

| Group | MPL treatment (μg)[a] | SSS-III-specific PFC/spleen[b] |
|---|---|---|
| A | | 4.134 ± 0.039 (13,617; $n$ = 18) |
| B | | 3.320 ± 0.049 (2,089; $n$ = 18) |
| C | 50 (days −1, 0) | 3.753 ± 0.108 (5,659; $n$ = 9) |
| D | 50 (days 0, 1) | 4.021 ± 0.060 (10,486; $n$ = 10) |

[a] BALB/cByJ mice were given a single injection (i.p.) of 0.005 μg of SSS-III (except mice in group A) 3 days before immunization (i.p.) with 0.5 μg of SSS-III (all groups). MPL was given (i.p.) on days −1 and 0, or on days 0 and 1 relative to immunization with 0.5 μg of SSS-III.

[b] $Log_{10}$ SSS-III-specific PFC per spleen ± SEM for $n$ mice 5 days after immunization (i.p.) with 0.5 μg of SSS-III; geometric means (antilogs) are given in parentheses.

the net result was not much different from that obtained with a single injection of 50 μg of MPL given on day 0 (Table 1; groups B versus E). By contrast, the administration of two injections of 50 μg of MPL on days 0 and 1 completely eliminated low-dose paralysis (Table 2; groups B versus D, $P < 0.05$); here, the resulting PFC response was of the same magnitude as that of the unprimed immunized controls (Table 2; groups A versus D, $P > 0.05$).

It should be noted that no SSS-III-specific PFC response could be detected in the majority (>95%) of mice 5 and 8 days after priming with a subimmunogenic dose (0.005 μg) of SSS-III. If mice primed with 0.005 μg of SSS-III were given 50 μg of MPL 3 days after priming, no SSS-III-specific PFC could be detected 5 days after treatment with MPL (data not shown). Thus, the increased response of primed mice given MPL at the time of immunization (Table 1) cannot be attributed to an augmented antibody (PFC) response to the priming dose (0.005 μg) of SSS-III. In previous studies (23) it has been shown that treatment with LPS causes the appearance of greater numbers of antigen-specific background PFCs (polyclonal activation) in the spleens of mice previously exposed to subimmunogenic amounts of antigen to generate detectable immunological memory. Since prior exposure to subimmunogenic or immunogenic doses of purified SSS-III fails to generate memory (10), SSS-III-specific PFCs are not usually found in the spleens of mice given amounts of LPS capable of stimulating significant polyclonal activation with respect to the antibody response to other antigens (23).

Significant decreases in the degree of low-dose paralysis expressed were also noted for mice given two lower doses of MPL (10 or 25 μg) on days 0 and 1 (Table 3; groups C and D versus group B, $P < 0.05$); the resulting PFC responses elicited after treatment with these lower doses of MPL were still less than those of unprimed immunized controls (Table 3; groups C and D versus group A, $P < 0.05$).

TABLE 3. Effect of time of administration and dose of MPL on the expression of low-dose paralysis to SSS-III

| Group | MPL treatment (μg)[a] | SSS-III-specific PFC/spleen[b] |
|---|---|---|
| A | | 4.431 ± 0.077 (26,992; $n$ = 7) |
| B | | 3.478 ± 0.158 (3,006; $n$ = 8) |
| C | 10 (days 0, 1) | 4.107 ± 0.099 (12,798; $n$ = 8) |
| D | 25 (days 0, 1) | 4.211 ± 0.058 (16,257; $n$ = 8) |

[a] BALB/cByJ mice were given a single injection (i.p.) of 0.005 μg of SSS-III (except mice in group A) 3 days before immunization (i.p.) with 0.5 μg of SSS-III (all groups). MPL was given (i.p.) on days 0 and 1 relative to immunization with 0.5 μg of SSS-III.

[b] $Log_{10}$ SSS-III-specific PFC per spleen ± SEM for $n$ mice 5 days after immunization (i.p.) with 0.5 μg of SSS-III; geometric means (antilogs) are given in parentheses.

Results of previous studies (2) have indicated that low-dose paralysis does not arise immediately upon exposure to a subimmunogenic dose of SSS-III; rather, it requires a latent (induction) period during which the degree of unresponsiveness expressed increases progressively until maximal levels are attained, 2 to 3 days after priming. The existence of such a latent phase, therefore, permits one to examine the effect of treatment with MPL on the induction of low-dose paralysis. To this end, groups of mice were given a single injection (i.p.) of 50 μg of MPL on the day of priming with 0.005 μg of SSS-III (day 0) or on days 1, 2, or 3 after priming; all mice were immunized with 0.5 μg of SSS-III 3 days after low-dose priming with 0.005 μg of SSS-III, and the effect of treatment with MPL on the degree of low-dose paralysis induced was assessed 5 days after immunization. The results obtained (Fig. 1) indicate that treatment with 50 μg of MPL had no effect ($P > 0.05$) on the degree of low-dose paralysis induced when given on the day of or the day after priming with 0.005 μg of SSS-III, i.e., days 0 and 1, respectively; however, low-dose paralysis was reduced significantly ($P < 0.05$) when MPL was given on day 2 or 3 after priming. Similar results were obtained with 25 μg of MPL, although the degree to which low-dose paralysis was decreased, although significant, was not as great as that noted at corresponding time intervals when 50 μg of MPL was used (data not shown).

**Effect of treatment with MPL on the magnitude of the antibody response to SSS-III.** Groups of mice were given (i.p.) a single injection of 5 to 50 μg of MPL at the time of immunization (i.p.) with an optimally immunogenic dose (0.5 μg) of SSS-III; the magnitude of the SSS-III-specific PFC response elicited was determined 5 days after immunization and was compared with that in immunized mice not given MPL. The administration of 5, 10, 25, or 50 μg of MPL at the time of immunization with 0.5 μg of SSS-III did not alter the magnitude of the SSS-III-specific PFC response made (Table 4; $P > 0.05$ for all comparisons between MPL-treated mice and controls). Although no effect ($P > 0.05$) was noted when 25 or 50 μg of MPL was given 1 day after immunization with 0.5 μg of SSS-III (day 1), significant enhancement ($P < 0.05$)

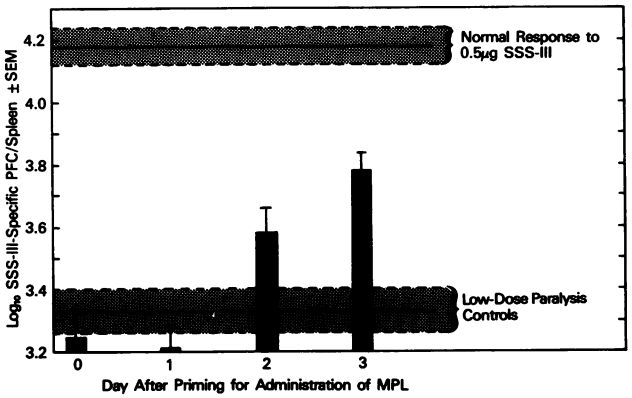


FIG. 1. Effect of giving 50 μg of MPL at different times after priming with 0.005 μg of SSS-III on the induction and expression of low-dose paralysis. Values shown are the means ± SEM for groups of similarly treated mice. Shaded areas designate the mean ± SEM for primed and unprimed immunized controls.

TABLE 4. Effect of MPL on the magnitude of the PFC response of mice to 0.5 μg of SSS-III

| Amt (μg) of MPL given[a] | SSS-III-specific PFC/spleen[b] |
|---|---|
| 0 | 4.306 ± 0.077 (20,253; $n$ = 7) |
| 5 | 4.150 ± 0.101 (14,118; $n$ = 7) |
| 10 | 4.309 ± 0.061 (20,354; $n$ = 7) |
| 25 | 4.158 ± 0.054 (14,396; $n$ = 8) |
| 50 | 4.226 ± 0.059 (16,838; $n$ = 8) |

[a] MPL was given (i.p.) at the time of immunization of BALB/cByJ mice with 0.5 μg of SSS-III.

[b] $Log_{10}$ SSS-III-specific PFC per spleen ± SEM for $n$ mice 5 days after immunization (i.p.) with 0.5 μg of SSS-III; geometric means (antilogs) are given in parentheses.

was evident when 25 or 50 μg of MPL was given 2 days after immunization with SSS-III (Table 5).

Groups of thymus-bearing (*nu/+*) and athymic nude (*nu/nu*) mice were given 50 μg of MPL 2 days after immunization with 0.5 μg of SSS-III, and the magnitude of the SSS-III-specific PFC response produced was compared with that of immunized *nu/+* and *nu/nu* mice not treated with MPL. The results obtained (Table 6) indicate that *nu/+* and *nu/nu* mice do not differ ($P > 0.05$) in their ability to make an antibody response to SSS-III, as expected (8, 19, 27, 28). Treatment with MPL increased the magnitude of the antibody response in *nu/+* mice ($P < 0.05$), but not in *nu/nu* mice ($P > 0.05$). This indicates that the enhancement produced following treatment with MPL is T-cell dependent and not due to a stimulatory or mitogenic effect of MPL upon B cells capable of responding to SSS-III.

We next examined the possibility that the increased antibody response to SSS-III in mice treated with MPL might be due to a stimulatory effect on MPL on B cells, which would be enhanced in the presence of T cells. This was not the case. Athymic *nu/nu* mice immunized with 0.5 μg of SSS-III were given 50 μg of MPL 2 or 3 days after immunization; then they were assayed for numbers of non-antigen-specific IgM-secreting PFCs 5 days after immunization. The results obtained (Table 7) indicate that, in the absence of T cells, significant ($P < 0.05$) polyclonal activation was evident 3 days after treatment with MPL, even though MPL did not increase the magnitude of the antibody response to SSS-III (Table 6). Also, MPL was found to be mitogenic for spleen cells from mice immunized with SSS-III; although the effects observed were dependent upon the dose of MPL as well as the numbers of cells used, they were T-cell independent (Table 8). The data in Table 8 suggest that the proliferative response of spleen cells from *nu/nu* mice is greater than that of *nu/+* mice; however, this may be due simply to differences between *nu/nu* and *nu/+* mice in the ratio of B cells to T cells per $10^5$ nucleated spleen cells. It should be noted that in both of these experiments, the polyclonal and mitogenic activities of MPL were examined with spleen cells from mice immunized with SSS-III to approximate the experimental conditions under which the other experiments described in this study were performed.

TABLE 5. Enhancement of the antibody response in mice given MPL at different times after immunization with 0.5 μg of SSS-III

| Day MPL given[a] | SSS-III-specific PFC/spleen after MPL was given at doses (μg) of[b]: | |
|---|---|---|
| | 25 | 50 |
| 1 | 4.471 ± 0.058 (27,575) | 4.499 ± 0.075 (31,585) |
| 2 | 4.685 ± 0.099 (48,383) | 5.017 ± 0.068 (104,006) |

[a] Day relative to the time of immunization (day 0) on which MPL was given (i.p.).

[b] $Log_{10}$ SSS-III-specific PFC per spleen ± SEM for groups of eight mice 5 days after immunization (i.p.) with 0.5 μg of SSS-III; geometric means (antilogs) are given in parentheses. The value for controls, which consisted of 13 mice immunized (i.p.) with 0.5 μg of SSS-III but not given MPL, was 4.375 ± 0.051 (geometric mean, 23,714).

TABLE 6. Inability of MPL to increase the magnitude of the antibody response to SSS-III in athymic nude (*nu/nu*) mice

| Mice | Amt (μg) of MPL given[a] | SSS-III-specific PFC/spleen[b] |
|---|---|---|
| *nu/+* | | 3.865 ± 0.069 (7,329; $n$ = 11) |
| *nu/+* | 50 | 4.417 ± 0.100 (26,132; $n$ = 9) |
| *nu/nu* | | 3.935 ± 0.071 (8,600; $n$ = 9) |
| *nu/nu* | 50 | 3.952 ± 0.092 (8,963; $n$ = 8) |

[a] MPL was given (i.p.) 2 days after immunization (i.p.) with 0.5 μg of SSS-III.

[b] $Log_{10}$ SSS-III-specific PFC per spleen ± SEM for $n$ mice 5 days after immunization (i.p.) with 0.5 μg SSS-III; geometric means (antilogs) are given in parentheses.

**Effect of treatment with MPL on the expression of amplifier and helper T-cell activity.** In other studies (4, 31) it has been shown that the administration of ConA 2 days after immunization with SSS-III results in a substantial increase in the magnitude of the antibody response to SSS-III; such enhancement is T-cell dependent and is mediated by amplifier T cells. To assess the effects of treatment with MPL on the expression of amplifier T-cell activity, groups of mice were given (i.p.) a single injection of 100 μg of MPL at the time of treatment with 300 μg of ConA; the magnitude of the SSS-III-specific PFC response produced was determined 5 days after immunization with 0.5 μg of SSS-III and compared with that of immunized mice given ConA but not MPL. Treatment with 300 μg of ConA resulted in significant enhancement of the SSS-III-specific PFC response, as expected (Table 9; groups A versus B, $P < 0.05$); however, treatment with MPL did not decrease the degree of ConA-induced enhancement elicited (Table 9; groups B versus C, $P > 0.05$). In another experiment (data not shown), treatment with 100 μg of MPL did not reduce ConA-induced enhancement when given 1 day after the administration of 300 μg of ConA ($P > 0.05$).

To assess the effects of treatment with MPL on the expression of helper T-cell activity, groups of mice were pretreated (primed) with a subimmunogenic dose (0.005%) of carrier (horse erythrocytes) to generate carrier-specific helper T cells. Three days later, they were given a single injection of conjugate (SSS-III–horse erythrocytes), with or without treatment with MPL; and the magnitude of the SSS-III-specific PFC response was determined 3 days after

TABLE 7. Increase in the number of IgM-secreting PFCs in the spleens of athymic nude (*nu/nu*) mice treated with 50 μg of MPL

| Day MPL given[a] | IgM-secreting PFC/spleen[b] |
|---|---|
| 0 | 5.201 ± 0.055 (159,033) |
| 2 | 5.568 ± 0.091 (369,974) |
| 3 | 5.341 ± 0.040 (219,059) |

[a] MPL (50 μg) was given (i.p.) 2 or 3 days after immunization (i.p.) with 0.5 μg of SSS-III.

[b] $Log_{10}$ IgM-secreting PFC per spleen ± SEM for 5 mice, 5 days after immunization (i.p.) with 0.5 μg of SSS-III; geometric means (antilogs) are given in parentheses.

TABLE 8. Proliferative response of spleen cells from thymus-bearing (*nu*/+) and athymic (*nu*/*nu*) mice to MPL

| Cell source | No. of cells/well ($10^5$) | [$^3$H]thymidine incorporation (cpm ± SEM) after the following amt (μg) of MPL was added/well[a]: | | | |
|---|---|---|---|---|---|
| | | 0 | 0.62 | 2.5 | 10 |
| *nu*/+ mice | 1.25 | 115 ± 23 | 362 ± 120 | 341 ± 70 | 385 ± 66 |
| | 2.50 | 214 ± 35 | 799 ± 100 | 1,023 ± 162 | 1,319 ± 250 |
| | 5.00 | 443 ± 50 | 2,227 ± 196 | 4,884 ± 729 | 8,320 ± 729 |
| | 10.00 | 1,550 ± 153 | 6,019 ± 348 | 15,104 ± 1,811 | 31,405 ± 3,703 |
| *nu*/*nu* mice | 1.25 | 200 ± 31 | 564 ± 106 | 2,173 ± 509 | 2,100 ± 611 |
| | 2.50 | 400 ± 88 | 1,262 ± 97 | 3,043 ± 306 | 5,577 ± 244 |
| | 5.00 | 551 ± 91 | 3,148 ± 247 | 12,132 ± 976 | 18,126 ± 2,741 |
| | 10.00 | 1,042 ± 115 | 7,346 ± 919 | 22,184 ± 2,541 | 45,324 ± 8,393 |

[a] [$^3$H]thymidine incorporation after culture in the presence of different amounts of MPL; quadruplicate cultures were used for each determination.

the administration of conjugate. The results obtained (Table 10) indicate that priming with carrier (horse erythrocytes) results in a substantial SSS-III-specific PFC response in mice immunized with a marginally immunogenic dose of conjugate (SSS-III–horse erythrocytes), as expected (Table 10; groups A versus B, $P < 0.05$); such a response is due to the generation of carrier-specific helper T cells (4, 15, 16, 32). Treatment with 100 μg of MPL did not decrease the magnitude of the helper T-cell-dependent response produced (Table 10; groups B versus C, $P > 0.05$).

## DISCUSSION

Since it was first reported that suppressor and amplifier T cells act in an opposing manner to control the magnitude of the antibody response to SSS-III (3, 12), much information has been obtained on (i) the cell surface antigens, (ii) the mode of action, and (iii) the kinetics for the induction and expression of the activities of these regulatory T cells (2, 7, 30, 32, 33, 44, 45). Consequently, this well-characterized experimental model system provides an excellent means for assessment of the influence of various treatments, immunomodulating agents, or both on the function of such cells. It has been used with advantage to reveal malfunctions in the immune system of autoimmune NZB mice (4, 33).

Although both suppressor and amplifier T cells are activated during the course of a normal antibody response to an optimally immunogenic dose of SSS-III (6, 44), the phenomenon of low-dose paralysis is a case in which the inhibitory effects of suppressor T cells predominate. Kinetic studies indicate that significant unresponsiveness can be detected as early as 12 h after priming with a single injection of a subimmunogenic dose of SSS-III; thereafter, the degree of unresponsiveness expressed increases progressively with time until maximal levels are attained 48 to 72 h after priming (2). Such unresponsiveness persists for at least 2 months, is antigen specific, and is mediated by Lyt-2$^+$, L3T4$^-$ suppressor T cells (2, 4, 10, 33). In the present study, MPL was without effect when given during the early inductive phase of low-dose paralysis; i.e., at the time of, or 1 day after, priming; however, the degree of low-dose paralysis expressed was reduced significantly when MPL was given 2 to 3 days after priming, i.e., at or near the time of maximal unresponsiveness (Fig. 1). This suggests that MPL acts preferentially on activated, rather than resting or precursor, suppressor T cells, to reduce the degree of unresponsiveness expressed. At this time, we do not know whether MPL kills suppressor T cells or alters their metabolic activity for a finite period of time; this is being investigated.

In other studies (43) it has been shown that suppressor T cells proliferate after low-dose priming, resulting in an increase in the pool of suppressor T cells capable of being activated upon subsequent immunization. The increased pool size, and perhaps the potential to become activated more quickly upon subsequent immunization, accounts for the unresponsiveness observed. The kinetics for the activation of suppressor T cells during the course of an antibody response to an optimally immunogenic dose of SSS-III are similar to those noted for the development of low-dose paralysis (30). Therefore, if MPL acts mainly on activated suppressor T cells, one would expect (i) MPL to be more effective in reducing the degree of low-dose paralysis expressed when given after the immunization of previously primed mice, and (ii) treatment with MPL to result in enhancement of the antibody (PFC) response of unprimed mice when given 2 days after immunization; this was indeed the case (Tables 2 to 5). Furthermore, such enhancement is T-cell dependent and not due simply to a stimulatory effect of MPL on B cells capable of responding to SSS-III (Tables

TABLE 9. Effect of treatment with MPL on ConA-induced enhancement of the PFC response to 0.5 μg of SSS-III

| Group | Treatment (μg)[a] | | SSS-III-specific PFC/spleen[b] |
|---|---|---|---|
| | ConA | MPL | |
| A | | | 4.288 ± 0.064 (19,426; $n$ = 10) |
| B | 300 | | 5.139 ± 0.087 (137,815; $n$ = 10) |
| C | 300 | 100 | 5.239 ± 0.097 (173,193; $n$ = 10) |

[a] ConA and MPL were given (i.p.) 2 days after immunization (i.p.) with 0.5 μg of SSS-III.

[b] $Log_{10}$ SSS-III-specific PFC per spleen ± SEM for $n$ BALB/cByJ mice 5 days after immunization (i.p.) with 0.5 μg of SSS-III; geometric means (antilogs) are given in parentheses.

TABLE 10. Effect of treatment with MPL on the expression of helper T-cell activity

| Group | MPL treatment (μg)[a] | SSS-III-specific PFC/spleen[b] |
|---|---|---|
| A | | 3.459 ± 0.117 (2,877; $n$ = 10) |
| B | | 4.786 ± 0.068 (61,043; $n$ = 9) |
| C | 100 | 4.920 ± 0.043 (83,231; $n$ = 9) |

[a] Mice were pretreated or primed (i.v.) with 0.2 ml of 0.005% horse erythrocytes (except mice in group A) 3 days before immunization (i.v.) with 0.2 ml of 1% SSS-III–horse erythrocytes (all groups). MPL was given (i.p.) at the time of immunization with SSS-III–horse erythrocytes.

[b] $Log_{10}$ SSS-III-specific PFC per spleen ± SEM for $n$ mice 3 days after immunization (i.v.) with 0.2 ml of 1% SSS-III–horse erythrocytes; geometric means (antilogs) are given in parentheses.

6 to 8). It should be noted that, since MPL is a small molecule (average molecular weight, 1,718), it is likely to be cleared from the circulation within a relatively short period of time after injection. Consequently, some of the effects produced after the administration of relatively large doses (50 to 100 μg) of MPL to primed mice at the time of immunization (Tables 1 and 2) may be attributed to the presence of residual amounts of MPL at the time that suppressor T cells become activated. The results of the present study indicate that multiple injections of small doses of MPL, given at appropriate times, may be more effective than a single large dose in inactivating suppressor T cells (Table 3).

The ability of MPL to increase the magnitude of the antibody response when given 2 days after immunization with SSS-III (Table 5) allows us to assume that such enhancement is due to the inactivation of suppressor T cells, thereby allowing the effects of amplifier T cells, which are known to be activated during the course of a normal antibody response to SSS-III, to be more fully expressed. There is no evidence to indicate that such enhancement is due to T-cell-dependent polyclonal activation of B cells by MPL (Tables 7 and 8); the failure of MPL to decrease ConA-induced enhancement (Table 7), which is mediated by activated amplifier T cells (4, 31), is consistent with such a view and indicates that amplifier T-cell activity may not be reduced by the doses of MPL used.

Both amplifier and helper T cells exert a positive influence on the magnitude of the antibody response; however, several observations indicate that these cells represent functionally distinct subpopulations of T cells. These have been summarized in detail elsewhere (4). Since helper T cells are not required for the initiation of a normal antibody response to SSS-III (8, 19, 27, 28), the effects produced by amplifier T cells can be more readily discerned; this would be more difficult to do and would require more extensive experimental manipulations, in the case of a helper T-cell-dependent antibody response (35). The immunization of carrier-primed mice with a marginally immunogenic dose of SSS-III bound to the same carrier provides a special case in which one is able to examine the effects of MPL on an antibody response to SSS-III that requires helper T-cell activity. The administration of a relatively large dose (100 μg) of MPL to carrier-primed mice at the time of immunization with SSS-III bound to the carrier did not decrease the magnitude of the antibody response made (Table 10). Since helper T cells act mainly during the first 24 h after immunization (36), it is reasonable to conclude that treatment with MPL does not reduce the expression of helper T-cell activity.

The ability of MPL to inactivate suppressor T cells, but not amplifier and helper T cells, is a unique phenomenon and suggests that MPL might be used with good advantage as an adjuvant or immunomodulating agent; this merits serious consideration, since phase I clinical trials indicate that large doses of MPL are relatively nontoxic to humans (47). The results of ontogeny studies show that suppressor T-cell activity is fully developed early in life, well before the appearance of amplifier T-cell activity (34); thus, MPL might be used to improve the effectiveness of vaccines in young children who otherwise might not be able to mount an adequate antibody response against certain bacterial infections (18, 37). MPL might also be useful, in combination with other therapeutic agents, in the treatment of various immunodeficiency syndromes (e.g., acquired immune deficiency syndrome) in which a decrease in helper T cells is associated with a concomitant increase in the percentage and absolute numbers of suppressor T cells (22, 24). Although it has long been known that bacterial endotoxins (LPSs) cause necrosis of tumors (42), results of recent studies indicate that (i) endotoxin-induced tumor regression is independent of tumor necrosis and is mediated by tumor-sensitized $L3T4^+$, $Ly1^+2^-$ T cells (20), and that (ii) treatment with LPS prevents the generation of suppressor T cells, thereby increasing the ability to reject transplantable tumors (17). In these situations, elimination of the inhibitory effects of suppressor T cells by treatment with nontoxic MPL might promote the development of a more effective immune response against tumor cells. All of these issues require further study.

In previous studies it has been shown that priming with a subimmunogenic dose of some bacterial LPS results in the development of immunological memory (5), whereas priming with other types of LPS results in the development of unresponsiveness which appears to be mediated by T cells (21). This suggests that not all preparations of LPS may possess the same immunomodulatory properties and that the MPL derived from various preparations of LPS may differ in its effect upon suppressor T cells. The significance of such differences to virulence as well as the development of protective immunity in the host remains to be evaluated.

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## LITERATURE CITED

1. **Baker, P. J., D. F. Amsbaugh, P. W. Stashak, G. Caldes, and B. Prescott.** 1981. Regulation of the antibody response to type III pneumococcal polysaccharide. Rev. Infect. Dis. **3**:332–341.
2. **Baker, P. J., D. F. Amsbaugh, P. W. Stashak, G. Caldes, and B. Prescott.** 1982. Direct evidence for the involvement of thymus-derived (T) suppressor cells in the expression of low-dose paralysis to type III pneumococcal polysaccharide. J. Immunol. **128**:1059–1062.
3. **Baker, P. J., R. F. Barth, P. W. Stashak, and D. F. Amsbaugh.** 1970. Enhancement of the antibody response to type III pneumococcal polysaccharide in mice treated with anti-lymphocyte serum. J. Immunol. **104**:1313–1315.
4. **Baker, P. J., M. B. Fauntleroy, P. W. Stashak, K. L. McCoy, and T. M. Chused.** 1986. Increased amplifier T cell activity in autoimmune NZB mice and its possible significance in the expression of autoimmune disease. Immunobiology **171**:400–411.
5. **Baker, P. J., J. R. Hiernaux, P. W. Stashak, and J. A. Rudbach.** 1985. Cyclic development of immunological memory to bacterial lipopolysaccharide. Infect. Immun. **48**:1–6.
6. **Baker, P. J., and B. Prescott.** 1979. Regulation of the antibody response to pneumococcal polysaccharides by thymus-derived (T) cells: mode of action of suppressor and amplifier T cells, p. 67–105. *In* J. A. Rudbach and P. J. Baker (ed.), Immunology of bacterial polysaccharides. Elsevier/North-Holland Publishing Co., New York.
7. **Baker, P. J., B. Prescott, P. W. Stashak, and D. F. Amsbaugh.** 1971. Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular level. III. Studies on the average avidity of the antibody produced by specific plaque-forming cells. J. Immunol. **107**:719–724.
8. **Baker, P. J., N. D. Reed, P. W. Stashak, D. R. Amsbaugh, and B. Prescott.** 1973. Regulation of the antibody response to type III pneumococcal polysaccharide. I. Nature of regulatory cells. J. Exp. Med. **137**:1431–1441.
9. **Baker, P. J., and P. W. Stashak.** 1969. Quantitative and qualitative studies on the primary antibody response to pneumococcal polysaccharide at the cellular level. J. Immunol. **103**:1342–

1348.
10. **Baker, P. J., P. W. Stashak, D. F. Amsbaugh, and B. Prescott.** 1971. Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular level. I. Dose-response studies and the effect of prior immunization on the magnitude of the antibody response. Immunology **20**:469–481.
11. **Baker, P. J., P. W. Stashak, D. F. Amsbaugh, and B. Prescott.** 1971. Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular level. II. Studies on the relative rate of antibody synthesis and release by antibody-producing cells. Immunology **20**:481–493.
12. **Baker, P. J., P. W. Stashak, D. F. Amsbaugh, B. Prescott, and R. F. Barth.** 1970. Evidence for the existence of two functionally distinct types of cells which regulate the antibody response to type III pneumococcal polysaccharide. J. Immunol. **105**: 1581–1583.
13. **Baker, P. J., P. W. Stashak, and B. Prescott.** 1969. The use of erythrocytes sensitized with purified pneumococcal polysaccharides for the assay for antibody and antibody-producing cells. Appl. Microbiol. **17**:422–426.
14. **Behling, U. H., and A. Nowotny.** 1982. Immunostimulation by LPS and its derivatives, p. 165–179. *In* H. Friedman, T. W. Klein, and A. Szentivanyi (ed.), Immunomodulation by bacteria and their products. Plenum Publishing Corp., New York.
15. **Braley-Mullen, H., and G. C. Sharp.** 1974. Conversion of type III pneumococcal polysaccharide low responders to high responders by immunization with a thymus dependent form of antigen. Cell. Immunol. **12**:49–60.
16. **Byfield, P.** 1972. Increased plaque-forming cell response to pneumococcal polysaccharide type III-coated sheep erythrocytes after priming with erythrocytes but not with polysaccharide. Cell. Immunol. **3**:616–622.
17. **Chun, M., and M. K. Hoffmann.** 1987. Combination immunotherapy of cancer in a mouse model: synergism between tumor necrosis factor and other defense systems. Cancer Res. **47**:115–118.
18. **Cowan, M. H., A. J. Ammann, D. W. Wara, V. M. Howie, L. Schulz, N. Doyle, and M. Kaplan.** 1978. Pneumococcal polysaccharide immunization in infants and children. Pediatrics **62**:721–727.
19. **Davies, A. J. S., R. L. Carter, E. Leuchars, V. Wallis, and F. M. Dietrick.** 1970. The morphology of immune reactions in normal, thymectomized, and reconstituted mice. III. Response to bacterial antigens Salmonella flagellar antigen and pneumococcal polysaccharide. Immunology **19**:945–957.
20. **Digiacomo, A., and R. J. North.** 1987. Subtherapeutic numbers of tumour-sensitized L3T4$^+$, Ly1$^+$2$^-$ T cells are needed for endotoxin to cause regression of an established immunogenic tumour. Immunology **60**:367–373.
21. **Elkins, K. L., P. W. Stashak, and P. J. Baker.** 1987. Mechanisms of specific immunological unresponsiveness to bacterial lipopolysaccharides. Infect. Immun. **55**:3093–3102.
22. **Fahey, J. L., H. Prince, M. Weaver, J. Groopman, B. V. Visschev, K. Schwartz, and R. Detels.** 1984. Quantitative changes in T helper or T suppressor/cytotoxic lymphocyte subsets that distinguish acquired immune deficiency syndrome from other immune subset disorders. Am. J. Med. **76**:95–100.
23. **Fauntleroy, M. B., J. A. Rudbach, B. Prescott, and P. J. Baker.** 1987. Increased activation of antigen-primed or memory B cells by bacterial lipopolysaccharide. Cell. Immunol. **107**:263–272.
24. **Giorgi, J. V., P. G. Nishanian, J. Schmid, L. E. Hultin, H.-L. Cheng, and R. Detels.** 1987. Selective alterations in immunoregulatory lymphocyte subsets in early HIV (human T-lymphotropic virus type III/lymphadenopathy-associated virus) infection. J. Clin. Immunol. **7**:140–150.
25. **Gottlieb, C. F.** 1974. Applications of transformations to normalize the distribution of plaque-forming cells. J. Immunol. **113**:51–57.
26. **Gronowicz, E., A. Coutinho, and F. Melchers.** 1976. A plaque assay for all cells secreting Ig of a given type or class. Eur. J. Immunol. **6**:588–598.
27. **Howard, J. G., G. H. Christie, B. M. Courtenay, E. Leuchars, and A. J. S. Davies.** 1971. Studies on immunological paralysis. VI. Thymic-independence of tolerance and immunity to type III pneumococcal polysaccharide. Cell. Immunol. **2**:614–626.
28. **Humphrey, J. H., D. M. V. Parrot, and J. East.** 1964. Studies on globulin and antibody production in mice at birth. Immunology **7**:419–436.
29. **Johnson, A. G., S. Gaines, and M. Landy.** 1956. Studies on the O-antigen of *Salmonella typhosa*. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. J. Exp. Med. **103**:225–246.
30. **Jones, J. M., D. F. Amsbaugh, P. W. Stashak, B. Prescott, P. J. Baker, and D. W. Alling.** 1976. Kinetics of the antibody response to type III pneumococcal polysaccharide. III. Evidence that suppressor cells function by inhibition of the recruitment and proliferation of antibody-producing cells. J. Immunol. **116**: 647–656.
31. **Markham, R. B., N. R. Reed, P. W. Stashak, B. Prescott, D. F. Amsbaugh, and P. J. Baker.** 1977. Effect of concanavalin-A on lymphocyte interactions involved in the antibody response to type III pneumococcal polysaccharide. II. Ability of suppressor T cells to act on both B cells and amplifier T cells to limit the magnitude of the antibody response. J. Immunol. **119**:1163–1168.
32. **Markham, R. B., P. W. Stashak, B. Prescott, D. F. Amsbaugh, and P. J. Baker.** 1978. Selective sensitivity to hydrocortisone of regulatory functions that determine the magnitude of the antibody response to type III pneumococcal polysaccharide. J. Immunol. **121**:829–834.
33. **McCoy, K. L., P. J. Baker, P. W. Stashak, and T. M. Chused.** 1985. Two defects of old New Zealand black mice are involved in the loss of low-dose paralysis to type III pneumococcal polysaccharide. J. Immunol. **135**:2438–2442.
34. **Morse, H. C., III, B. Prescott, S. S. Cross, P. W. Stashak, and P. J. Baker.** 1976. Regulation of the antibody response to type III pneumococcal polysaccharide. V. Ontogeny of factors influencing the magnitude of the plaque-forming cell response. J. Immunol. **116**:279–287.
35. **Muirhead, D. Y., and G. Cudkowicz.** 1978. Subpopulations of splenic T cells regulating an antihapten antibody response. I. Helper and amplifier T cells. J. Immunol. **120**:579–585.
36. **Muirhead, D. Y., and G. Cudkowicz.** 1978. Subpopulations of splenic T cells regulating an antihapten antibody response. II. Distinct functions of, and sequential requirement for, helper and amplifier cells. J. Immunol. **121**:130–137.
37. **Peltola, H., H. Käyhty, M. Virtanen, and P. H. Mäkelä.** 1984. Prevention of *Haemophilus influenza* type b bacteremia infections with the capsular polysaccharide vaccine. N. Engl. J. Med. **310**:1561–1566.
38. **Ribi, E.** 1984. Beneficial modification of the endotoxin molecule. J. Biol. Response Mod. **3**:1–9.
39. **Ribi, E., J. Cantrell, T. Feldner, K. Myers, and J. Peterson.** 1986. Biological activities of monophosphoryl lipid A, p. 9–13. *In* L. Levine, P. F. Bonventre, J. A. Morello, F. D. Silver, and H. C. Wu (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
40. **Ribi, E., J. L. Cantrell, K. Takayama, N. Qureshi, J. Peterson, and H. O. Ribi.** 1984. Lipid A and immunotherapy. Rev. Infect. Dis. **6**:567–572.
41. **Ribi, E., J. L. Cantrell, K. Takayama, H. O. Ribi, K. R. Myers, and N. Qureshi.** 1986. Modulation of humoral and cell-mediated immune responses by a structurally established non-toxic lipid A, p. 407–420. *In* A. Szentivanyi and H. Friedman (ed.), Immunobiology and immunopharmacology of bacterial endotoxins. Plenum Publishing Corp., New York.
42. **Shear, M. J.** 1944. Chemical treatment of tumors. IX. Reactions of mice with primary subcutaneous tumors of injection of a hemorrhagic-producing bacterial polysaccharide. J. Natl. Cancer Inst. **4**:461–476.
43. **Stashak, P. W., C. E. Taylor, G. Caldes, B. Prescott, and P. J. Baker.** 1983. Cyclic expression of low-dose paralysis. Cell. Immunol. **77**:143–149.
44. **Taylor, C. E., P. W. Stashak, G. Caldes, B. Prescott, T. E. Chused, A. Brooks, and P. J. Baker.** 1983. Activation of antigen-specific suppressor T cells by B cells from mice immunized with

type III pneumococcal polysaccharide. J. Exp. Med. **158**:703–717.

45. **Taylor, C. E., P. W. Stashak, J. Chiang, W. M. Leiserson, G. Caldes, B. Prescott, and P. J. Baker.** 1984. Characteristics of amplifier T cells involved in the antibody response to the capsular polysaccharide of type III *Streptococcus pneumoniae*. J. Immunol. **132**:3103–3108.
46. **Tomai, M. A., L. E. Solem, A. G. Johnson, and E. Ribi.** 1987. The adjuvant properties of nontoxic monophosphoryl lipid A in hyporesponsive and aging mice. J. Biol. Response Mod. **6**:99–107.
47. **Vosika, G. J., C. Barr, and D. Gilbertson.** 1984. Phase I study of intravenous modified lipid A. Cancer Immunol. Immunother. **18**:107–112.