Differential Sensitivity of CD8+ Suppressor and Cytotoxic T Lymphocyte Activity to Bacterial Monophosphoryl Lipid A

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Treatment with a preparation of monophosphoryl lipid A, known to be capable of abolishing the expression of CD8+ suppressor T cell activity generated during the antibody response to type III pneumococcal polysaccharide (SSS-III) (8, 9), was found to have no adverse effect upon either induction or expression of CD8+ cytotoxic T lymphocyte activity specific for influenza A virus antigens. This suggests that suppressor T cells and cytotoxic T lymphocytes represent functionally distinct subsets of CD8+ T cells which can be differentiated on the basis of their sensitivities to inactivation by monophosphoryl lipid A.

Recent studies showed that treatment with bacterial monophosphoryl lipid A (MPL) abrogates the expression, but not the induction, of the activity of CD8+ suppressor T cells (Ts) elicited in response to type III pneumococcal polysaccharide (SSS-III) (8, 9). This occurs without adversely influencing functions mediated by CD4+ amplifier T cells (Ta) (8, 9) and helper T cells (Th) (8). It results in the reversal of low-dose immunological paralysis, a form of antigen-specific unresponsiveness mediated by CD8+ Ts (5, 6, 30), and enables Ta activity to be more fully expressed, as evidenced by an increased antibody response to SSS-III (8, 9). Prior in vitro treatment with MPL also abolishes the capacity of spleen cell suspensions containing Ts activity to transfer suppression to recipient mice immunized with SSS-III (15, 19). These observations imply that Ts, once activated, acquire a cell surface receptor for MPL and/or possess a biochemical pathway that is sensitive to being blocked or inactivated by MPL (5, 8, 19). MPL, therefore, can be considered to be a potent immunomodulator, since by eliminating the inhibitory effects of Ts it can greatly increase the expression of an immune response.

In the present work, the effect of in vitro and in vivo treatment with MPL on both the induction and expression of the activity of influenza virus-specific CD8+ cytotoxic T lymphocytes (Tc) was examined to determine whether the aforementioned effects of MPL are selective for Ts or whether they also apply to CD8+ Tc. The results obtained indicate that treatment with amounts of MPL known to abrogate the expression of Ts activity has no adverse effect on either induction or expression of Tc activity. Thus, Ts and Tc appear to represent functionally distinct subsets of CD8+ T cells that can be distinguished by means of their sensitivities to MPL.

MATERIALS AND METHODS

Mice. Female BALB/cByJ (H-2b) and (C57BL/6 × SJL)F1 (H-2b/H-2b) mice, 6 to 8 weeks old, were obtained from the Jackson Laboratories, Bar Harbor, Maine.

MPL. MPL isolated from Salmonella minnesota R595 [MPL(SM)] was purchased from Ribi ImmunoChem Research, Inc., Hamilton, Mont. The results of several preliminary experiments revealed that its immunomodulatory properties are the same as those described for MPL extracted from Salmonella typhimurium (8, 9). Lyophilized MPL(SM) was reconstituted to a concentration of 1 mg/ml in sterile distilled water containing 0.2% triethylamine. It was warmed to 45°C, mixed thoroughly, and then sonicated (15 s) to obtain a stock solution which was stored at 4°C until used. The stock solution was diluted to the desired concentration of MPL(SM) in either saline (for injection) or RPMI 1640 medium (for in vitro experiments) just before use. It should be noted that all of the experiments described in this report were conducted with the same lot of MPL (lot 039-171), known to be capable of eliminating Ts activity; similar results can be obtained with other lots of MPL(SM).

Antigen and the detection of antibody-producing cells. The immunological properties of the SSS-III used and the method by which it was prepared have been described previously (5, 10–13). For immunization, mice were given a single intraperitoneal (i.p.) injection of an optimally immunogenic dose (0.5 μg) of SSS-III in saline. Numbers of splenic antibody-producing plaque-forming cells (PFC) making antibody specific for SSS-III (SSS-III-specific PFC) provided a measure of the antibody response made at peak, i.e., 5 days after immunization. SSS-III-specific PFC making antibody of the immunoglobulin M class (>90% of all PFC found [11]) were detected by a slide version of the technique of localized hemolysis-in-gel by using indicator sheep erythrocytes coated with SSS-III by the CrCl method (14). Corrections were made for the small numbers of background sheep erythrocyte-specific PFC detected so that only values for SSS-III-specific PFC are considered. The values obtained (SSS-III-specific PFC per spleen), which are log normally distributed (22), are expressed as the geometric mean (antilog) of the mean log10 number of SSS-III-specific PFC per spleen for groups of similarly treated mice. This provides an accurate measure of the total antibody response elicited, since (i) SSS-III-specific PFC are detected only in the spleens of immunized mice (2), and (ii) the numbers of SSS-III-specific PFC per spleen are directly related to the magnitude of the serum antibody response (2, 23). Student's t test was used to evaluate the significance of the differences found; differences were considered to be significant when probability (P) values <0.05 were obtained.
**Cell lines.** The cell lines used in this work were the T cell lymphoma RMA (H-2k) and the mastocyteoma P815 (H-2b); the former was kindly provided by K. Karre, Karolinska Institute, Stockholm, Sweden, and the latter has been maintained in our laboratory for several years. Both cell lines were grown in RPMI 1640 medium ( Gibco Inc., Grand Island, N.Y.) supplemented with glutamine (100 mM), gentamicin (50 μg/ml), and 7.5% heat inactivated fetal bovine serum (HyClone, Logan, Utah).

**Virus and peptides.** A stock solution of influenza A virus, PR/8/34, containing about 2,000 hemagglutination units (HAU) per ml in anlatico fluid, was diluted 1:5 in phosphate-buffered saline (pH 7.4). Mice were inoculated (i.p.) with 0.5 ml of the dilute solution to elicit a primary immune response to the virus.

Peptides corresponding to amino acid residues 365 to 380 (peptide 365-380), as well as to residues 147 to 158 of the nucleoprotein (NP) of PR/8/34 virus, were synthesized by the Biological Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Peptides 365-388 and 147-158 mimic the NP epitopes recognized by Tc in association with D0 and K0 class I major histocompatibility complex molecules, respectively (29, 31).

**Generation of Tc.** Tc were generated in vitro as described previously (32). Briefly, spleen cell suspensions were prepared from mice inoculated at least 2 weeks earlier with 200 HAU of PR/8/34 virus. Then, 2 × 106 cells per ml were restimulated with either NP peptide (1 μg/ml) or syngeneic spleen cells infected with PR/8/34 (104 per ml) for 7 days. It should be noted that stimulation with NP peptide induces exclusively anti-NP-specific Tc, whereas stimulation with virus-infected cells results in the development of several populations of Tc recognizing different viral proteins, including NP (3).

**Preparation of target cells for use in cytotoxicity assays.** Target cells were infected with PR/8/34 and labeled with 51Cr as described previously (32). Chromium-labeled target cells were sensitized with NP peptide for use in cytotoxicity assays by adding to the cultures 10 μl of tissue culture medium containing peptide, giving a final peptide concentration of 10−6 M. Peptide was present in culture throughout the cytotoxicity assay to generate a secondary cytotoxic response directed against the epitope of that peptide.

**Cytotoxicity assay.** Tc generated after 7 days of culture were washed and then resuspended in Iscove's Dulbecco's modified Eagle's medium supplemented with glutamine (100 mM), gentamicin (50 μg/ml), β-mercaptoethanol (5 × 10−5 M), and 7.5% heat inactivated fetal bovine serum (3, 32). Then, 100 μl of culture material containing different numbers of effector Tc was added to 100 μl of medium containing peptide or virus-treated 51Cr-labeled target cells (104) in 96-well round-bottom plates. After a 4-h incubation at 37°C, 100 μl of medium was removed and the amount of 51Cr released was measured with a gamma counter. The amount of 51Cr released spontaneously was determined after the incubation of target cells alone, whereas values for the total amount of 51Cr capable of being released were determined after incubating labeled target cells in the presence of a dilute (1:6) solution of saturated cetramide in saline. Each result obtained is presented as the mean (± the standard deviation) percentage of specific release of 51Cr for triplicate cultures at several effector-to-target (E/T) cell ratios according to the formula [(cpm of released sample - cpm of spontaneous release)/(cpm of total release - cpm of spontaneous release)] × 100. In all experiments, the percentage of spontaneous release of 51Cr by target cells was less than 20% of the total release value.

It has been established that CD8+ Tc, but not CD4+ T cells or B cells, generated after virus infection (29) recognize NP peptides 147-158 and 365-380; also, it has been established that the secondary cytotoxic response generated after exposure to these peptides is epitope specific as well as class I restricted (3, 32).

**RESULTS**

**Effect of treatment with MPL(SM) on the expression of Ts activity involved in the antibody response.** BALB/cByJ mice were pretreated (primed) with a single injection (i.p.) of a subimmunogenic dose (5 ng) of SSS-III; 3 days later, they were immunized (i.p.) with an optimal dose (0.5 μg) of SSS-III, and the magnitude of the antibody (SSS-III-specific PFC) response produced was determined 5 days after immunization. The results obtained were compared with those of unprimed immunized controls.

Priming with 5 ng of SSS-III resulted in the development of significant (P < 0.001) immunological unresponsiveness, as expected (Table 1); it has been established that such unresponsiveness is antigen specific, persists for several weeks to months after priming with a single injection of SSS-III, and is mediated by CD8+ T cells that become fully activated 3 days after priming (5, 30). If primed mice are given (i.p.) 25 μg of MPL(SM) with 0.5 μg of SSS-III on the day of immunization (day 0) and one day after immunization (day +1), unresponsiveness is abolished; i.e., the resulting response does not differ from that of unprimed immunized controls (P > 0.05; Table 1).

The ability to transfer antigen-specific low-dose paralysis by CD8+ T cells (6, 30) attests to the fact that such unresponsiveness is mediated by Ts and enables one to examine directly the ability of MPL(SM) to abrogate Ts activity by using a preparation of MPL derived from S. typhimurium [MPL(ST)], as described in another study (15). The in vitro treatment of primed spleen cells with extremely small amounts (5 pg, 5 ng, or 5 μg) of MPL(SM) ablated their ability to transfer suppression to recipient mice immunized with SSS-III (19). All of these findings are in complete accord with those obtained in previous studies (8, 15, 19) conducted with MPL(ST). Therefore, the inactivation of Ts activity is a general property of MPL.

**Effect of in vitro treatment with MPL(SM) on the expression of Tc activity.** (C57BL/6 × SJL)F1 mice were inoculated (i.p.) with 200 HAU of PR/8/34. Two weeks later, spleen cell

### Table 1. Effect of treatment with MPL(SM) on the expression of low-dose immunological paralysis to SSS-III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Priming</th>
<th>Immunization</th>
<th>MPL(SM)</th>
<th>SSS-III-specific PFC/spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>4.101 ± 0.036 (12,630)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2.610 ± 0.300 (407)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4.026 ± 0.042 (10,611)</td>
</tr>
</tbody>
</table>

* Mice were given 5 ng of SSS-III (i.p.) 3 days before i.p. immunization with 0.5 μg of SSS-III.

* 25 μg of MPL(SM) was given (i.p.) on day 0 and on day +1 relative to immunization.

* Log10 SSS-III-specific PFC per spleen ± standard error of the mean for groups of 10 BALB/cByJ mice, 5 days after immunization with 0.5 μg of SSS-III; geometric means (antilogs) are in parentheses.
suspensions were prepared which were cultured in vitro for 7 days in the presence of 1 μg of NP peptide 365–380 per ml to elicit a secondary cytotoxic response directed against NP peptide 365–380. During this time, effector cells either were cultured in the continuous presence of different amounts (0.05 ng, 5 ng, 0.5 μg) of MPL(SM) or they were briefly treated with different amounts of MPL(SM) and then washed prior to culture [MPL(SM)-pulsed cells]. After 7 days in culture, the cells were assayed for cytotoxic activity at several E/T cell ratios by using ⁵¹Cr-labeled RMA target cells sensitized with NP peptide 365–380; controls consisted of ⁵¹Cr-labeled target cells not sensitized with NP peptide 365–380. The data of Fig. 1 show that in vitro treatment with different amounts of MPL(SM) had no effect on the expression of Tc activity. This was the case for effector cells either cultured in the presence of MPL(SM) or pulsed with MPL(SM) just prior to culture. No ⁵¹Cr release was detected when effector cells were added to target cells not sensitized with NP peptide 365–380 (data not shown); this indicates that the cytotoxic response detected is specific for NP peptide 365–380.

Effect of in vivo treatment with MPL(SM) on the induction of Tc activity. BALB/cByJ mice were inoculated (i.p.) with 200 HAU of PR/8/34 and were given either two or four i.p. injections of MPL(SM) at different times relative to infection. Here, MPL(SM) was given on days 1 and 2 after infection (two injections) or on days 1, 2, 6, and 7 after infection (four injections). Two weeks after infection, spleen cell suspensions were prepared which were then cultured in vitro for 7 days in the presence of stimulator cells infected with PR/8/34; the cultures were then assayed for cytotoxic activity at several E/T cell ratios, using ⁵¹Cr-labeled P815 target cells sensitized with NP peptide 147–158 or infected with PR/8/34. The results obtained were compared with those for infected mice not treated with MPL(SM) to evaluate the effect of treatment with MPL(SM) on the induction or expression of Tc activity in vivo.

The data of Fig. 2 show that treatment with two injections of MPL(SM) during the first 7 days after inoculation with virus had no discernible effect on the induction of Tc activity; however, treatment with four injections of MPL(SM), two during the first week and two during the second week after inoculation with virus, resulted in a marked increase in the degree of Tc activity generated. The increase was most pronounced with target cells sensitized with NP peptide 147–158, although it also was apparent with target cells infected with PR/8/34. The specificity of cytotoxicity was affirmed by the fact that no ⁵¹Cr release was noted.

![FIG. 1. Effect of in vitro treatment with MPL(SM) on the expression of Tc activity specific for NP peptide 365–380. (a) Cells cultured in the continuous presence of MPL(SM); (b) cells pulsed with MPL(SM) prior to culture.](http://iai.asm.org/)

![FIG. 2. Effect of in vivo treatment with MPL(SM) on the primary induction of Tc activity. (a) Cultures were stimulated with PR/8/34 and then assayed against NP peptide 147–158-sensitized ⁵¹Cr-labeled target cells; (b) cultures were stimulated with PR/8/34 and then assayed against PR/8/34-sensitized ⁵¹Cr-labeled target cells.](http://iai.asm.org/)
with 51Cr-labeled P815 target cells not sensitized with NP peptide 147–158 or PR/8/34 (data not shown).

**DISCUSSION**

Although CD8+ Ts have been shown to act in a negative and antigen-specific manner to influence the magnitude of the antibody response to SSS-III (4, 5, 10), the major differences between Ts and Tc, which likewise are CD8+ (4) and are generated during the immune response to viral antigens and alloantigens, remain to be more fully defined.

The ability of either Tc cell-depleting agents or the infusion of cell suspensions possessing CD4+ Ta activity to reverse the inhibitory effects of Ts in vivo indicates that Ts activated during the immune response to SSS-III are regulatory rather than cytotoxic in their mode of action (reviewed in reference 7). Because of these and other observations, it has been proposed that the magnitude of the antibody response to SSS-III is controlled by two types of regulatory T cells (Ts and Ta) that act in an opposing and competitive manner on B cells to limit the extent to which antigen-stimulated B cells proliferate after immunization (reviewed in references 4, 5, and 10). All experimental data acquired to date are in accord with such a homeostatic model for regulating the magnitude of the antibody response.

Fitch et al. (21) reported that the ability of irradiated spleen cells from mixed leukocyte cultures (MLC) to inhibit the generation of Tc activity is due mainly to the inactivation of alloantigen-bearing stimulator cells by residual Tc present in the irradiated cell suspensions used; this results in a marked decrease in the degree of Tc activity generated when such cells were added at the initiation of MLC. Here, both the suppression noted and the cytotoxic activity expressed are considered to be mediated by the same population of T cells acting under different experimental circumstances. By contrast, the work of Al-Adra and Pilarski (1) provided convincing evidence to indicate that the suppression induced during primary (first-step) MLC reactions is not mediated by Tc. Rather, it is the result of a distinct regulatory process engendered by the ability of both responder and inhibitory cells (Tc and Ts, respectively) to recognize and respond to the same alloantigens on the surface of stimulator cells. The capacity of cyclosporin A to block the activation of Tc, but not the generation of noncytolytic alloantigen-specific Ts, affirms that suppression and cytotoxicity are mediated by separate populations of T cells (16).

Also, the ability to induce alloantigen-specific Ts activity in the absence of Tc activity (28), as well as the ability to differentiate between Ts and Tc on the basis of CD11b and CD28 phenotype (25) or sensitivity to inactivation by monoclonal antibodies specific for antigen-specific suppressor factors (18), provides additional support for the view that Ts indeed represent a separate subset of CD8+ T cells.

In the present work, treatment with a preparation of MPL known to abolish the expression of CD8+ Ts activity involved in the antibody response to SSS-III (Table 1 (11) had no adverse effect on either the in vitro expression (Fig. 1) or the in vivo induction (Fig. 2) of CD8+ Tc activity specific for viral antigen. This indicates that Ts and Tc represent functionally distinct subsets of CD8+ T cells which can be differentiated on the basis of their sensitivity to inactivation by MPL. The ability of MPL to increase the frequency or degree of Tc activity expressed (Fig. 2) provides additional support for such an activity view; it showed that MPL pressure to eliminate the inhibitory effects of Ts known to be generated during the course of cytotoxic immune responses (1, 6–18, 25, 28), permits the effects produced by Tc to be more fully expressed.

The results of the present work (Fig. 2) indicate that little or no MPL-sensitive suppression is evident during the first week after the inoculation of virus; however, such suppression appears to be expressed during the second week after virus infection, as evidenced by an increase in the degree of Tc activity expressed in mice given four injections of MPL. The ability of MPL to increase the degree of Tc activity generated during the primary (Fig. 2), but not the secondary (Fig. 1), response to viral antigens suggests that such suppression may have a decisive effect during the initial stage of clonal expansion of Tc. Also, since previous studies (8, 9) showed that MPL acts mainly on activated rather than resting or precursor Ts, it is conceivable that greater increases in Tc activity might be achieved by giving the same or larger doses of MPL at different times after exposure to viral antigens. Clearly, more detailed and systematic studies which are beyond the scope of the present work are required to establish the experimental conditions for maximizing the effects of MPL for this immune response.

The ability of MPL to abrogate the expression of Ts activity, without adversely influencing the expression of Tc as well as other Tc activities (8, 9), has profound implications with respect to increasing the development of T cell-mediated immunity. There is much evidence to indicate that Ts activity is generated not only during the course of a normal antibody response (5) but also during UV radiation-induced carcinogenesis (20) and during the immune response to tumor antigens (24, 26, 27); in the latter case, the development of Ts activity, albeit of different antigenic phenotype, has been shown to inhibit the regression of tumor growth mediated by Tc. Obviously, under such conditions, MPL might be used with great advantage to promote or increase the efficiency of tumor immunity. This is being investigated in collaborative studies with other investigators.

**REFERENCES**


