Specific T-cell tolerance may reflect selective activation of lymphokine synthesis

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ABSTRACT Selective T-cell unresponsiveness, as measured by interleukin 2 (IL-2) synthesis upon challenge with antigen, was induced in SJL mice by ovalbumin (OVA) in incomplete or complete Freund's adjuvant administered i.p. or s.c. Ten days later, the mice were given booster injections of 100 μg of OVA/complete Freund's adjuvant. On day 20, lymph node and spleen cells were challenged in vitro with serial dilutions of OVA. There was an antigen-specific dose-dependent drop in IL-2 production in lymph node T cells. Conversely, 100 μg of OVA upregulated IL-4 and, to a lesser extent, interferon γ (IFN-γ) production, particularly by spleen T cells. Altogether, these data indicate that the drop in IL-2 production and T-cell proliferation, and the upregulation of IL-4 and IFN-γ production, are complex manifestations of an evolving T-cell response. The maturation of the T-cell response leads to the production of different patterns of lymphokines, which may be significantly affected, as desired, by dosage, timing, and route of immunization, as well as by the choice of adjuvants.

We have previously observed that specific T-cell tolerance to ovalbumin (OVA) in SJL mice, as measured by the secretion of interleukin 2 (IL-2) by lymph node T cells upon antigen challenge in vitro, could result from the injection of OVA in incomplete Freund's adjuvant (IFA) before immunization with the antigen in complete Freund's adjuvant (CFA). We reported (1) that the amounts of OVA were critical but the site (i.p. or s.c.) of antigen injection was not. Furthermore, the deficient T-cell response to OVA as evidenced by a decrease in IL-2 synthesis, and cell replication upon antigen challenge, was not associated with a decrease in the production of anti-OVA antibodies.

To extend these observations and to further define the type of specific T-cell tolerance achieved by this protocol, we have evaluated the development of T helper cells (Th1/Th2) in the tolerant mice and specifically the secretion, upon antigen challenge in vitro, of IL-4 and interferon γ (IFN-γ) by lymph nodes and spleen T cells of tolerant mice.

The deficiency in IL-2 synthesis was often associated with an increase in IL-4 and a moderate decrease in IFN-γ. Moreover, a comparative analysis of the effect of tolerance induction in lymph node and spleen T cells was very informative. More marked increases in IL-4 and in IFN-γ production were observed by spleen than lymph node T cells upon challenge with OVA in vitro.

The tolerizing injection of OVA in IFA or CFA is apparently highly selective in reducing IL-2 responses and proliferation of lymph node T cells and is associated with a shift to the synthesis of other lymphokines, indicative of the complex regulation of the T-cell response in peripheral lymphoid organs.

MATERIALS AND METHODS

Reagents. Crystallized chicken OVA was purchased from Sigma.

Induction of T-Cell Tolerance. Female SJL (H-2b) mice (The Jackson Laboratory) 6–18 weeks old were used for all experiments. To induce tolerance, animals were given the indicated amounts of OVA by injection i.p. or s.c. in IFA (Difco) or CFA (H37 Ra; Difco). Control animals were immunized with phosphate-buffered saline (PBS) in IFA or CFA. Nine to 11 days later, animals were immunized at the base of the tail with 100 μg of OVA in CFA. Nine to 11 days later, mice were sacrificed, and lymph nodes were collected. For antiserum collection, mice were bled and sacrificed on day 21–28 after CFA immunization.

Cell Preparation and Culture. Lymphocyte suspensions were teased from superficial and deep inguinal lymph nodes or spleens with a 10-ml tissue grinder, and debris was removed by filtration through a nylon mesh. Cell suspensions were washed twice before resuspension in culture medium [RPMI 1640 medium (Irvine Scientific)/1% heat-inactivated normal mouse serum (Organon Teknika)/4 mM L-glutamine/20 mM Heps/1 mM nonessential amino acids/50 mM 2-mercaptoethanol/40 μg of gentamicin per ml/100 units of penicillin per ml/100 μg of streptomycin per ml/0.25 μg of fungizone per ml]. Five to 6 × 10⁵ cells per well in the presence or absence of OVA were brought to a final vol of 2 μl in flat-bottom microculture plates. The lymph node and spleen T-cell cultures were incubated in a humidified 5% CO₂/95% air atmosphere at 37°C. One hundred microliters of supernatant was removed after 1, 3, or 4 days; frozen; and then assayed for IL-2, IFN-γ, or IL-4, respectively. IL-2 was assayed as described (2) by using the IL-2-dependent cell line HT-2. IFN-γ was assayed as described (3) by growth inhibition of the murine B-lymphoma WEHI 279 cell line and expressed as percentage inhibition of replication. IL-4 was assayed as described (4) by using the IL-4-dependent cell line CT-4S. For T-cell proliferation assay, cultures were pulsed on day 3 with 1 μCi of [³H]thymidine (1 Ci = 37 GBq) and harvested the next morning. The specificity of the IL-2, IL-4, and IFN-γ assays was confirmed in experiments with 11B11 antibody (anti-IL-4) (5), S4B6 antibody (anti-IL-2) (6), and XMG1.2 antibody (anti-IFN-γ) (7).

RESULTS

Selective T-Cell Tolerance in Lymph Node Cells After Previous Immunization i.p. or s.c. with OVA in IFA. Selective T-cell tolerance, as evidenced by inhibition of IL-2 synthesis by lymph node T cells of tolerant mice upon antigen challenge in vitro, was observed after either i.p. or s.c. immunization with OVA in IFA or CFA (1). To further investigate the immune status of these tolerant mice, the production of IFN-γ and IL-4 and T-cell proliferation upon antigen challenge in vitro were also assayed.

On day 10 after tolerance induction with OVA in IFA or CFA, mice were immunized at the base of the tail with 100 μg of OVA in CFA. On day 20, the mice were sacrificed and cell

Abbreviations: OVA, ovalbumin; IL, interleukin; IFN, interferon; Th, T helper.

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suspensions of lymph node and spleen cells were cultured for 24 h to measure IL-2 production, for 72 h to measure IFN-γ synthesis, or for 96 h to assay IL-4 production and T-cell proliferation. Table 1 presents the tolerization and immunization protocols as well as the timing of the various assays for all the experimental groups.

As previously reported (1), earlier immunization with OVA in IFA either i.p. or s.c. causes a significant decrease in proliferative response and IL-2 synthesis by lymph node T cells upon antigen challenge in vitro. These effects are considerably stronger with 1 mg than with 100 μg of OVA (Fig. 1).

The tolerizing effect of previous immunization is much less apparent on the synthesis of IFN-γ and barely reduces the synthesis of IL-4. In fact, after tolerization with both 0.1 and 1 mg of OVA s.c., the lymph node cells of tolerized mice show a significant increase in IL-4 synthesis upon antigen challenge compared with the control group.

Failure of Spleen T Cells to Express Specific T-Cell Tolerance After Previous Immunization i.p. or s.c. with OVA in IFA. Contrary to the selective tolerance observed in lymph node T cells following the above immunization protocol, evidenced by markedly reduced IL-2 synthesis upon antigen challenge, the spleen T cells of tolerized animals showed little (at 1 mg of OVA s.c.) or no effect of specific tolerance as evidenced by IL-2 synthesis (Fig. 2). There were no marked effects of this treatment on IFN-γ production either. However, IL-4 synthesis by spleen T cells was strongly up regulated by the effect of the earlier immunization, which in this case could be clearly interpreted as classical priming, as was the case also for the increased level of specific anti-OVA antibodies on days 20 and 30 after initial immunization (data not shown).

Selective Tolerization with OVA in CFA Instead of IFA. To investigate the possible effect of the adjuvant, the identical tolerizing protocol described earlier was used with CFA instead of IFA and the results of these experiments are shown in Figs. 3 and 4 for the responses of lymph node T cells and spleen T cells, respectively.

The results were remarkably similar to those observed with IFA. Earlier immunization resulted in selective, dose-dependent marked suppression of IL-2 synthesis upon antigen challenge in lymph node cells but not in spleen cells. The increases in IL-4 responses by both lymph node and spleen cells were even more marked than when IFA was used as adjuvant.

DISCUSSION
Specific T-cell tolerance to OVA, as measured by a reduction in the proliferative response and IL-2 synthesis of lymph node T cells after antigen challenge, was shown to follow a primary response (1). The present study was designed to determine what parameters would induce T-cell tolerance versus a recall or "secondary T-cell response." For this purpose, we compared the effect of primary immunization with OVA/IFA and OVA/CFA administered i.p. or s.c. on T-cell tolerance induction by using several parameters of T-cell response.

Our previous study (1) and the data presented in this report clearly show that active immune responses are induced by both OVA/IFA and OVA/CFA at day 10 and are followed by a down regulation of IL-2 production by lymph node cells on day 20 after secondary challenge s.c. with 100 μg of OVA in CFA. Spleen cells in contrast were much less susceptible to suppression of IL-2 synthesis. Moreover, up regulation of IL-4, and to a much lesser extent of IFN-γ production by spleen cells, was observed in tolerized mice.

Our data indicate that spleen T cells are more resistant to the down regulation of IL-2 production and more sensitive to the induction of IL-4 responses as compared to lymph node T cells. One milligram of OVA was always more efficient in

Table 1. Experimental protocol

<table>
<thead>
<tr>
<th>Tolerization (IFA/CFA; s.c./i.p.)</th>
<th>Immunization</th>
<th>Culture*</th>
<th>Supernatant collection and assays</th>
</tr>
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<tr>
<td>Day 0</td>
<td>Day 10</td>
<td>Day 20</td>
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<tr>
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<td>100 μg OVA</td>
<td>IL-2</td>
<td>HT-2</td>
</tr>
<tr>
<td>PBS 1 mg OVA</td>
<td>NOTHING</td>
<td>IL-4</td>
<td>WEHI-279</td>
</tr>
<tr>
<td>PBS 1 mg OVA</td>
<td>NO TREATMENT</td>
<td>INF γ</td>
<td>CT-4S</td>
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<tr>
<td>PBS 1 mg OVA</td>
<td>T-CELL PROLIF</td>
<td>PULSE</td>
<td>HARVEST</td>
</tr>
</tbody>
</table>

* Lymph node and spleen cells.

FIG. 1. Immune status of lymph node T cells after tolerization i.p. or s.c. with OVA/IFA. Groups of SJL mice were tolerized either i.p. (A, C, E, and G) or s.c. (B, D, F, and H) with either PBS (squares) or 1 mg (circles) or 100 μg (triangles) of OVA in IFA (100 μl per mouse); on day 10, one group of mice was booster injected with 100 μg of OVA in CFA (100 μl per mouse) (solid symbols), and one group of mice was kept for controls (open symbols). On day 20, the mice were sacrificed and lymph node cells were collected. Duplicate microcultures (200 μl) with serial dilutions of OVA were prepared with 6 × 10⁶ lymph node T cells. Cultures were incubated for 24 h (IL-2) (A and B), 72 h (IFN-γ) (G and H), or 96 h (IL-4) (C and D) and T-cell proliferation (TCP) (E and F) at 37°C. Data are means of three representative experiments.

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tolerance induction (down regulation of IL-2 production) than 100 μg of OVA. Although it has been quite well demonstrated

that Th1 and Th2 subsets could down regulate one another (8–12), up regulation of both IL-4 and IFN-γ production by splenic T cells suggests that regulation of lymphokine production occurs at a gene expression level (as already demonstrated with a Th2 clone) (13) and is more subtle than the partial or total disappearance of a Tq subset. Our results are also in agreement with recent studies showing that cytokine secretion by recently activated CD4+ T cells does not usually follow a pure Th1 or Th2 pattern (14, 15). Therefore, it is tempting to speculate that lymph nodes have an environment favorable to the development of Th1 cells, whereas the splenic tissue is more compatible with the development of Th0 cells and/or an equal ratio of Th1 and Th2 cells.

The phylogeny between Th1 (IL-2, IFN-γ), Th2 (IL-4), and Th0 (IL-2, IL-4, and IFN-γ) subsets is still a matter of investigation. However, some immune responses seem to be dominated by a Th1 cytokine profile and others are dominated by a Th2 cytokine profile. For instance, in mice of most inbred strains such as C57BL/6, locally initiated infection with Leishmania major leads to production of Th1 cytokines and a strong cell-mediated immune response (16, 17). In contrast, helminth parasite infection induces the production of an elevated level of Th2 cytokines (18, 19).

A Th1 response has been often associated with a state of active immunity or tissue rejection; Th2 response is associated with permisivity or a state of active tolerance or suppression. This assumption has been more directly supported with the work of Lin et al. (20) showing that in the placenta Th2 cells dominate over the Th1 subset, suggesting that Th2 cells would explain the tolerance of the mother in regard to the fetus antigen.

However, some good evidence suggests that IL-4 and/or Th2 cytokines by themselves cannot always account for self tolerance. (i) Although arthritis is often ameliorated during pregnancy, lupus erythematosus is also often exacerbated in pregnant women (21). (ii) In contrast to Leishmania major infection for which Th1 cytokines lead to healing and Th2 cytokines lead to permisivity and death, helminth infection is cured by Th2 cytokines and Th1 cytokines exacerbate the disease (22). (iii) IL-4 increase in vitro has been reported by two laboratories (23,
24) in antigen-specific tolerant mice. In contrast, memory T cells have been found to be a potent source of IL-4 (25-27) and IFN-γ (25-29).

No difference between control mice and tolerant mice was seen in antibody production (data not shown). It has been recognized for a long time that B cells are more resistant than T cells to tolerance induction (30). Our study therefore suggests that this might be due to the refractoriness of splenic T cells to tolerance induction. Furthermore, the increase of IL-4 production by splenic T cells must be promoting the increase of antibody levels regularly seen in mice primed i.p. and challenged s.c., as compared to mice primed i.p. only (data not shown). Therefore, the increase of IL-4 and IFN-γ that we have seen may most likely reflect the activation of OVA-specific memory T cells.

Our data further illustrate how, in the course of an evolving T-cell immune response, the production of different patterns of lymphokines occurs in distinct peripheral lymphoid organs. These patterns may be significantly affected therapeutically by dosage, timing, and route of immunization as well as by the choice of adjuvant.

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