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Lack of Orally Induced Systemic Unresponsiveness in IFN-γ Knockout Mice

Mi-Na Kweon,* Kohtarou Fujihashi,* John L. VanCott,† Kazuo Higuchi,* Masafumi Yamamoto,** Jerry R. McGhee,‡ and Hiroshi Kiyono2*‡

Splenic T cells isolated from BALB/c mice that had been mucosally tolerized by oral administration of 25 mg of OVA revealed selective increases in IFN-γ production with impaired levels of IL-2, IL-4, IL-5, and IL-10. These mice possessed reduced splenic OVA-specific T cell proliferative and delayed-type hypersensitivity responses when compared with nontolerized controls. Further, OVA-specific IgG Ab responses in serum and the numbers of IgG Ab-forming cells in spleen were significantly diminished following systemic challenge with OVA in CFA. When IFN-γ-deficient (IFN-γ−/−) mice of the same genetic background were given an oral dose of 25 mg of OVA before systemic immunization, no reduction in OVA-specific IgG Ab responses in serum and spleen was seen. Furthermore, the serum IgG Ab responses were restricted to IgG1 and IgG2b subclasses. Interestingly, although IFN-γ−/− mice displayed a partial diminishment of T cell proliferative and delayed-type hypersensitivity responses to OVA, significant responses were still present when compared with the low responses noted in IFN-γ+/+ mice. In addition, OVA-specific T cells from IFN-γ−/− mice produced Th2-type cytokines (e.g., IL-4), which provided help for systemic OVA-specific serum IgG1 and IgG2b Ab responses. These findings clearly indicate a central role for IFN-γ in the induction and maintenance of mucosally induced tolerance. The Journal of Immunology, 1998, 160: 1687–1693.

Analysis of tandem cytokine production by murine CD4+ Th cell clones has provided direct evidence that Th cells can be divided into two distinct classes, Th1- and Th2-type cells, on the basis of the pattern of cytokines secreted (16). It is well established that CD4+ Th1-type cells secrete IFN-γ, IL-2, and lymphotoxin-α for cell-mediated immunity and delayed-type hypersensitivity (DTH),3 whereas CD4+ Th2-type cells preferentially secrete IL-4, IL-5, IL-6, IL-10, and IL-13, which provide help for humoral Ab responses of different isotypes (16–18). In view of the distinct characteristics displayed during the immune response (e.g., cell-mediated immunity or DTH vs Ab isotype/subclass responses), it seemed plausible that Th1- and Th2-type cells also play different roles in mucosally induced tolerance (19). Recent studies have suggested that Th1-type cells appear to be more susceptible to the induction of tolerance than Th2-type cells (20), as manifested by their diminished IgG2a responses and reduced IL-2 and IFN-γ production (21). Ongoing studies have shown that tolerance could be induced in IL-4 knockout mice that have a dominant Th1 with defective Th2 responses when a high oral dose of Ag was administered (22); however, the precise mechanisms for tolerance remain undefined.

In this study, we have adopted an experimental system of IFN-γ gene-disrupted (IFN-γ−/−) and normal (IFN-γ+/+) mice to elucidate the role of Th1 and Th2 cells in mucosally induced tolerance. When mice of these two types were given a high oral dose of OVA, clear differences in the level of tolerance induction of T and B cells were noted. Our results support a central role for IFN-γ in the induction of mucosally induced tolerance.

Materials and Methods

Mice

All BALB/c and C57BL/6 mice used in our experiments were obtained from The Jackson Laboratory (Bar Harbor, ME). Both IFN-γ−/− on a

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3 Abbreviations used in this paper: DTH, delayed-type hypersensitivity; AFC, Ab-forming cells; IFN-γ−/−, IFN-γ gene knockout; ELISPOT, enzyme-linked immunospot.
BALB/c background and IFN-γ−/− on a C57BL/6 background were used between the ages of 8 and 12 wk in our experiments. The BALB/c IFN-γ−/− mice were generously provided by Dr. Timothy A. Stewart of Gentech (San Francisco, CA; Ref. 23) and the C57BL/6 IFN-γ−/− mice were obtained from The Jackson Laboratory. The IFN-γ−/− mice were genotyped by PCR. All mice were provided sterile food and water ad libitum and maintained under pathogen-free conditions in a flexible Trexler isolator (Standard Safety Equipment Co., Palatine, IL).

**Immunization protocols**

To induce systemic unresponsiveness to OVA (Fraction V; Sigma Chemical Co., St. Louis, MO), mice were given 25 mg of OVA dissolved in 0.25 ml of PBS by gastric intubation. Control mice received PBS only. On the 7th and 21st days following intubation, mice were challenged by the s.c. route with 100 μg of OVA in 100 μl of CFA (Difco Laboratories, Detroit, MI) to ensure the development of mucosally induced tolerance. DTH responses and serum Ab titers were measured 7 days after the second s.c. immunization. In most studies, the experiment was terminated at this point for analysis of OVA-specific B and T cell responses.

**OVA-specific serum Abs by ELISA**

Anti-OVA Ab titers in serum samples were determined by ELISA as described previously (24). Briefly, Falcon Microtest III assay plates (Becton Dickinson, Oxnard, CA) were coated overnight at 4°C with 1 mg/ml of OVA in PBS. Blocking was done with 200 μl of 1% BSA in PBS for 1 h at 37°C. Serial dilutions of serum in 1% BSA/PBS were prepared and 100 μl was added per well in duplicate. The plates were incubated at 37°C for 4 h, the horseradish peroxidase-labeled anti-mouse μ, γ, and α heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added and incubated overnight at 4°C. For IgG subclass determinations, biotin-conjugated mAbs specific for IgG1, IgG2a, IgG2b, and IgG3 (PharMingen, San Diego, CA) and peroxidase-labeled anti-biotin Ab (Vector Laboratories, Inc., Burlingame, CA) were employed. Color was developed with 1.1 mM 3,3′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H2O2.

**Enumeration of Ab-forming cells by enzyme-linked immunospot (ELISPOT)**

An ELISPOT assay was used to detect OVA-specific IgM, IgG, and IgA Ab-forming cells (AFC) in spleen from immunized mice as described previously (24, 25). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore Corp., Bedford, MA) were coated with OVA (1 mg/ml) in PBS (100 μl/well) overnight at 4°C. Wells were blocked with PBS containing 5% BSA. After addition of appropriate dilutions of spleen cells in RPMI 1640 containing 10% heat-inactivated FCS to individual wells, the plates were incubated for 4 h at 37°C with 5% CO2. The AFC were detected with peroxidase-labeled anti-mouse μ, γ, and α-chain-specific Abs (Southern Biotechnology Associates) and visualized by adding the chromogenic substrate, 3-aminio-9-ethylcarbazole (Moss, Inc., Pasadena, CA). Spots representing AFC were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope Systems, Olympus, Lake Success, NY).

**In vitro assay for B cell unresponsiveness**

To simultaneously perform in vitro and in vivo experiments, spleen cells were isolated from orally treated IFN-γ−/− and IFN-γ+/- mice following a single systemic immunization. Splenic mononuclear cells were prepared by a standard protocol as described previously (24, 25). These mononuclear cells (5 × 105 cells/ml) were cocultured with OVA (1 mg/ml) for 5 days in vitro. Nonadherent cells were harvested and then subjected to the OVA-specific ELISPOT assay described above.

**Measurement of DTH responses**

A standard protocol for the measurement of DTH responses was employed in this study (26). Briefly, 10 μg of OVA in 20 μl of PBS were injected into the left ear pinna; the right ear pinna received PBS as a control. Ear swelling was measured 48 h later with a dial thickness gauge (Ozaki Mfg. Co., Ltd., Tokyo, Japan). The DTH response was expressed as the increase in ear swelling after OVA injection following subtraction of swelling in the control site injected with PBS.

**OVA-induced splenic T cell proliferation**

Splenic T cells were harvested by using a nylon wool column, and a single cell suspension was prepared in complete medium (RPMI 1640; Cellgro Mediatech, Washington, DC) containing 10% heat-inactivated FCS, HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Splenic T cells (4 × 106 cells/ml) were cultured with 1 mg/ml of OVA in the presence of T cell-depleted, irradiated (3000 rad) splenic feeder cells from naive BALB/c mice in 96-well or 24-well tissue culture plates (Corning Glass Works, Corning, NY) for proliferation assay and cytokine analysis, respectively (27). In some experiments, splenic and Peyer’s patch T cells from mice orally immunized with 25 mg of OVA alone were prepared by a combination of enzymatic digestion (24, 25) and iron wool column purification, and were then cultured as described above. To measure OVA-specific T cell proliferation, 0.5 μCi of tritiated [3H]thymidine (Amersham Corp., Arlington Heights, IL) was added for the final 18 h of a 4-day culture period. All T cell cultures were incubated at 37°C in a moist atmosphere of 5% CO2 in air.

**Cytokine-specific ELISA**

Cytokine levels in culture supernatants were determined by ELISA as previously described (26–29). Culture supernatants were collected at 48 h for IFN-γ and IL-2, and at 96 h for IL-4, IL-5, and IL-10 production. The immunoplates (Nunc, Inc., Naperville, IL) were coated with monoclonal anti-IFN-γ (R4-6A2), anti-IL-2 (JE66-1A12), anti-IL-4 (BVD4-1D11), anti-IL-5 (TRFK-5), or anti-IL-10 Ab (JES5-2A5) (PharMingen). After blocking with 3% BSA in PBS, serial twofold dilutions of standards and samples were added to duplicate wells and incubated overnight at 4°C. The wells were washed and incubated for 2 h with anti-IFN-γ (XMG1.2), anti-IL-2 (JE66-5H4), anti-IL-4 (BVD4-24G2), anti-IL-5 (TRFK-4), or anti-IL-10 (JES5-16E5) Ab, respectively. After incubation, peroxidase-labeled anti-biotin Ab (Vector Laboratories, Burlingame, CA) were added and washed. 1.1 mM 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H2O2. Standard curves were generated using mouse rIFN-γ, rIL-2, rIL-4, rIL-5 (Genzyme, Cambridge, MA), or rIL-10 (PharMingen).

**Statistics**

The significance of the differences between groups was evaluated by the Mann-Whitney U test for unpaired samples using a Statview II Program designed for Macintosh computers.

**Results**

**Lack of B cell unresponsiveness in IFN-γ−/− mice**

In our initial studies, we determined whether oral administration of 25 mg of OVA induced B cell unresponsiveness in IFN-γ−/− (BALB/c) mice by assessing OVA-specific serum Ab titers. Serum from IFN-γ−/− mice given OVA by the oral route possessed significantly lower levels of Ag-specific IgG Abs than did control mice given oral PBS before s.c. immunization with OVA in CFA (Fig. 1). Levels of OVA-specific IgG subclasses were also assessed and IgG1 was found to be the most abundant Ag-specific IgG subclass in the serum of mice given oral PBS following systemic challenge with OVA in CFA but was found in much lower levels in mice that had been fed OVA. In addition, the levels of serum OVA-specific IgG2a and IgG2b responses were also reduced in IFN-γ−/− mice orally immunized with OVA, but the differences were not statistically significant. Based on the profile of IgG subclass responses, Th2-dependent Ab responses (i.e., IgG1) appear to be more susceptible to tolerance induced by a high dose of OVA.

To directly examine the effect of Th1 and Th2 cells on mucosally induced tolerance, we investigated mice lacking Th1 cells due to the disruption of the IFN-γ gene. As shown in Figure 1, the magnitude of the OVA-specific Ab response was generally lower in IFN-γ−/− than in IFN-γ+/- mice. In contrast to the results for IFN-γ−/− mice, the levels of OVA-specific IgG Abs were essentially unchanged in sera from IFN-γ−/− mice given oral OVA or PBS. Further, IgG responses were restricted to IgG1 and IgG2b subclasses in both groups of IFN-γ−/− mice. Because the possibility existed that IFN-γ−/− mice might take longer periods for development of Ag-specific immune responses due to the lack of an appropriate Th cell environment, a longer interval of OVA-specific Ab responses was also examined (e.g., up to 4 wk after secondary systemic challenge). The levels of serum OVA-specific...
IgG Abs at 2 to 4 wk following systemic challenge were similar to those seen at the 1-wk interval (data not shown). These results further demonstrate that IFN-$\gamma^{-/-}$ mice orally immunized with a high dose of OVA do not develop reductions in OVA-specific IgG Ab titers in serum.

To ensure that intact B cell responses were maintained in IFN-$\gamma^{-/-}$ mice following oral immunization with a high dose of OVA, we next investigated the frequency of OVA-specific AFC in spleen of IFN-$\gamma^{-/-}$ or IFN-$\gamma^{+/+}$ mice orally immunized with OVA using an Ag-specific ELISPOT assay. The numbers of splenic OVA-specific IgG AFC were significantly decreased (13-fold) in IFN-$\gamma^{+/+}$ mice given oral OVA when compared with mice that received oral PBS (Table I). In contrast, though the levels of OVA-specific IgG AFC were slightly lower in the OVA-fed group than in that treated with PBS (Table I), the decrease was not statistically significant. An identical pattern of results was obtained when IFN-$\gamma^{-/-}$ mice of a different background (e.g., C57BL/6) were employed. These findings have provided new evidence that Ag-specific Ab unresponsiveness does not develop following oral administration of OVA in an environment of IFN-$\gamma$ deficiency.

To further confirm these findings, we performed an in vitro study in which spleen cells from OVA- or PBS-fed mice were cocultured with OVA. After cultivation with OVA for 5 days, splenic mononuclear cells were harvested and the Ag-specific ELISPOT assay was performed (Table II). OVA-specific IgG AFC were much less prevalent in spleens from OVA-fed IFN-$\gamma^{+/+}$ mice than in those from PBS-fed mice. In contrast, the frequency of IgG AFC was not significantly changed in IFN-$\gamma^{-/-}$ mice given oral OVA when compared with the PBS-fed group. Taken together, these results show that oral administration of a high dose of OVA elicits B cell unresponsiveness in normal IFN-$\gamma^{+/+}$ mice, but does not affect OVA-specific B cell responses of IFN-$\gamma^{-/-}$ mice.

Oral administration of a high dose of OVA induced partial T cell unresponsiveness in IFN-$\gamma^{-/-}$ mice

To determine the influence of a high dose of oral Ag on T cell responses in a situation of IFN-$\gamma$ deficiency, Ag-specific DTH responses were assessed after oral administration of OVA to IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice. Significantly lower Ag-specific DTH responses were noted in IFN-$\gamma^{+/+}$ mice orally immunized with OVA than in mice given oral PBS only (Table III). In contrast, the

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**FIGURE 1.** Analysis of OVA-specific IgG and IgG subclasses in serum of IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ mice orally immunized with 25 mg of OVA (○) or PBS (●) only, followed by s.c. immunization with OVA in CFA. The serum was obtained from five mice per group and tested individually. The results are expressed as the mean ± SE and show an example from three separate experiments. *, p < 0.05; **, p < 0.01 vs PBS orally administered group.

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**Table I.** The frequency of Ag-specific Ab-forming cells in spleen of IFN-$\gamma^{-/-}$ mice orally immunized with OVA

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Oral Immunization</th>
<th>Number of OVA-Specific AFC / $1 \times 10^6$ Cells $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-$\gamma^{+/+}$</td>
<td>PBS</td>
<td>10.8 ± 3.5</td>
</tr>
<tr>
<td>IFN-$\gamma^{-/-}$</td>
<td>OVA</td>
<td>6.3 ± 2.9</td>
</tr>
<tr>
<td>IFN-$\gamma^{-/-}$</td>
<td>PBS</td>
<td>27.5 ± 1.5</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-$\gamma^{+/+}$</td>
<td>PBS</td>
<td>13.5 ± 1.6</td>
</tr>
<tr>
<td>IFN-$\gamma^{-/-}$</td>
<td>OVA</td>
<td>27.3 ± 6.0</td>
</tr>
<tr>
<td>IFN-$\gamma^{-/-}$</td>
<td>PBS</td>
<td>17.0 ± 2.0</td>
</tr>
<tr>
<td>IFN-$\gamma^{-/-}$</td>
<td>PBS</td>
<td>11.5 ± 3.2</td>
</tr>
</tbody>
</table>

$^a$ For the assessment of splenic IgM, IgG, and IgA AFC, an ELISPOT assay was employed ($^{**}p < 0.01$ vs PBS orally immunized IFN-$\gamma^{-/-}$ mice).
Once it was established that oral administration of 25 mg of OVA induced cytokine responses (especially IgG1 subclass responses) and the number of OVA-specific AFCs in spleen of normal IFN-γ−/− mice but not in decreases in Ag-specific DTH responses were modest in OVA-fed IFN-γ−/− mice when compared with those of IFN-γ+/+ mice. The fact that Ag-specific DTH responses were observed in both groups of IFN-γ−/− mice (Table III) suggests that DTH responses can be induced and maintained even when Th1-type cells are deficient. Indeed, it was shown that cytokines other than IFN-γ, e.g., TNF-α, lymphotixin-α, and IL-8, may also play important roles in Ag-specific DTH responses (30, 31).

To further characterize OVA-specific T cell responses in IFN-γ−/− mice fed a high dose of OVA, we examined OVA-specific T cell proliferative responses in vitro. Splenic T cells isolated from IFN-γ+/+ mice orally immunized with OVA showed significantly lower Ag-specific T cell proliferative responses than did those from IFN-γ+/+ mice given PBS orally before systemic immunization (Table III). Reduced T cell proliferation was only observed in response to OVA, since Con A-stimulated proliferative responses were not affected in splenic T cells isolated from either IFN-γ−/− or IFN-γ+/+ mice given oral OVA or PBS (data not shown). On the other hand, although splenic T cells from IFN-γ−/− mice orally immunized with OVA showed decreased levels of OVA-specific proliferative responses when compared with IFN-γ−/− mice given PBS orally, the differences were not statistically significant (Table III). Taken together, these findings provide new evidence that oral administration of a high dose of OVA elicits only partial T cell unresponsiveness in IFN-γ−/− mice.

OVA-induced cytokine responses

Once it was established that oral administration of 25 mg of OVA resulted in the reduction of OVA-specific T cell proliferative responses in both IFN-γ+/+ and IFN-γ−/− mice, it seemed important to determine the effects of OVA on cytokine production patterns. Culture supernatants from OVA-stimulated splenic T cells were examined by ELISA for Th1- (e.g., IFN-γ and IL-2) and Th2-type cytokines (e.g., IL-4, IL-5, and IL-10). Much less IL-4, IL-5, and IL-10 was produced by OVA-stimulated T cells in culture supernatants from IFN-γ−/− mice orally immunized with OVA than by IFN-γ+/+ mice given oral PBS (Fig. 2). These findings are consistent with the fact that decreased serum IgG1 responses occur in IFN-γ−/− mice given oral OVA. Interestingly, predominant IFN-γ production was noted in culture supernatants from OVA-stimulated splenic T cells of orally tolerant IFN-γ−/− mice. To determine whether the increased IFN-γ production was due to a combination of oral OVA followed by OVA and CFA immunization, IFN-γ−/− mice were orally immunized with 25 mg of OVA alone, and T cells from spleen and Peyer’s patches of these mice were isolated 3 days later. Although T cells isolated from Peyer’s patches showed Ag-induced cytokine responses including significant amounts of IFN-γ synthesis, splenic T cells failed to produce cytokines. These results suggest that a combination of oral and systemic immunization elicits a dominant IFN-γ response by splenic T cells. These findings provide the first evidence that oral administration of a high dose of Ag elicits IFN-γ production by OVA-stimulated splenic T cells, and that this occurs despite the fact that T cell proliferative and DTH responses are reduced in orally tolerant IFN-γ−/− mice. Analysis of cytokine synthesis by Ag-stimulated splenic T cells from IFN-γ−/− mice orally immunized with OVA revealed low levels of IL-2, IL-4, IL-5, and IL-10 in culture supernatants when compared with those from mice orally administered PBS only. However, it is important to point out that although the levels of IL-4 were reduced in both IFN-γ−/− and IFN-γ+/+ mice orally immunized with OVA, higher IL-4 production was seen in IFN-γ−/− mice when compared with IFN-γ+/+ mice.

To examine a possible role for TGF-β in the induction and maintenance of tolerance to orally administered OVA, we measured levels of TGF-β in serum and culture supernatants harvested from orally treated IFN-γ−/− and IFN-γ+/+ mice. Interestingly, no significant differences were observed between the sera from the respective groups of IFN-γ−/− and IFN-γ+/+ mice (data not shown). Furthermore, TGF-β was not detected in culture supernatants of OVA-stimulated splenic T cells in vitro, suggesting that TGF-β may not have an effect on the induction of systemic unresponsiveness by a high dose of Ag.

### Discussion

This study has shown that oral administration of a high dose of OVA induces diminished levels of OVA-specific serum IgG (especially IgG1 subclass responses) and the number of OVA-specific AFCs in spleen of normal IFN-γ−/− mice but not in

### Table II. The frequency of OVA-specific Ab-forming cells in splenocyte cultures prepared from IFN-γ−/− mice orally immunized with OVA

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Oral Immunization</th>
<th>In Vitro Stimulation</th>
<th>Numbers of OVA-Specific AFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ+/+</td>
<td>PBS</td>
<td>Medium</td>
<td>IgM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVA</td>
<td>15.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVA</td>
<td>93.8 ± 6.3</td>
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<td></td>
<td></td>
<td>OVA</td>
<td>5.3 ± 2.1</td>
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<td></td>
<td></td>
<td>OVA</td>
<td>272.5 ± 12.5</td>
</tr>
<tr>
<td>IFN-γ−/−</td>
<td>PBS</td>
<td>Medium</td>
<td>13.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVA</td>
<td>557.5 ± 32.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVA</td>
<td>23.0 ± 6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVA</td>
<td>447.5 ± 27.5</td>
</tr>
</tbody>
</table>

*Whole spleen cells (5 × 10^6/ml) were cultured with 1 mg/ml of OVA or medium alone. After cultivation for 5 days, nonadherent cells were harvested and Ag-specific AFC were determined using an ELISPOT assay (**p < 0.01 vs PBS orally immunized IFN-γ−/− mice).

### Table III. Characterization of OVA-specific DTH and T cell proliferative responses of IFN-γ−/− mice orally immunized with OVA

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Oral Immunization</th>
<th>DTH (µm)*</th>
<th>Proliferation (cpm)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ+/+</td>
<td>PBS</td>
<td>227.0 ± 48.8</td>
<td>2384.8 ± 490.6</td>
</tr>
<tr>
<td></td>
<td>OVA</td>
<td>31.0 ± 25.8***</td>
<td>317.2 ± 224.8*</td>
</tr>
<tr>
<td>IFN-γ−/−</td>
<td>PBS</td>
<td>103.8 ± 40.9</td>
<td>2104.5 ± 448.0</td>
</tr>
<tr>
<td></td>
<td>OVA</td>
<td>32.0 ± 16.8**</td>
<td>1091.5 ± 216.8</td>
</tr>
</tbody>
</table>

*For the assessment of DTH responses, 20 µg of OVA in 20 µl of PBS or PBS only was injected into the left and right ear pinnas, respectively. Ear swelling was measured 48 h later and the DTH responses were expressed as the difference between the OVA- and PBS-injected ears (***p < 0.001, **p < 0.01 vs PBS orally immunized group).

* Splenic T cells (2 × 10^6 cells per well) were taken 1 week after the second s.c. immunization and cultured with or without 1 mg/ml of OVA in the presence of T cell-depleted and irradiated APCs from normal BALB/c mice (2.5 × 10^6 cells per well) for 4 days. During the last 18 h of incubation, 0.5 µCi of tritiated thymidine was added to each well. The results were expressed as cpm of the OVA-stimulated minus cpm of the medium only. Baseline proliferation of splenic T cells in medium only was under 200 cpm (*p < 0.05 vs PBS orally immunized group).
identically treated IFN-\(\gamma^{-/-}\) mice (Fig. 1, Tables I and II). Although OVA-induced proliferative responses of splenic T cells and DTH responses were decreased in both IFN-\(\gamma^{-/-}\) and IFN-\(\gamma^{-/-}\) mice following oral administration of OVA, decreases were more modest in IFN-\(\gamma^{-/-}\) than in IFN-\(\gamma^{-/-}\) mice, which exhibited complete suppression of proliferative and DTH responses (Table III). When the same experimental protocol was used in IL-4\(^{-/-}\) mice, both T and B cell unresponsiveness was observed after oral administration of OVA, as demonstrated by reduced OVA-specific T cell proliferation and DTH responses and markedly diminished OVA-specific serum IgG production (our manuscript in preparation). Indeed, it has been shown that Th2 cells are not required for the induction of mucosally induced tolerance since oral administration of 25 mg of OVA can elicit OVA-specific B and T cell unresponsiveness in IL-4\(^{-/-}\) mice (22). Taken together, these findings suggest that IFN-\(\gamma\)-producing Th1 cells are essential for the reduction of systemic B cell responses induced by a high dose of oral OVA.

Of interest was the finding that IFN-\(\gamma\) was the only cytokine demonstrating increased production in culture supernatants of OVA-stimulated splenic T cells from IFN-\(\gamma^{-/-}\) mice given oral OVA, even though the synthesis of IL-2 and IFN-\(\gamma\) and 4 days (for IL-4, IL-5, and IL-10) of incubation and analyzed by the respective cytokine-specific ELISA. Results are expressed as the mean \(\pm\) SE and show an example from three separate experiments containing five mice per group. *, \(p < 0.05\) vs PBS orally immunized group.

![FIGURE 2. Profiles of Th1- and Th2-type cytokine synthesis. A, Splenic T cells were isolated from IFN-\(\gamma^{-/-}\) and IFN-\(\gamma^{-/-}\) mice orally immunized with 25 mg of OVA (\(○\)) or PBS (\(□\)) only, followed by s.c. immunization with OVA in CFA. Splenic T cells (4 \(\times\) 10\(^6\) cells/ml) from the immunized mice were cultured with 1 mg/ml of OVA in the presence of T cell-depleted and irradiated splenic feeder cells (5 \(\times\) 10\(^6\) cells/ml) from naive BALB/c mice. B, IFN-\(\gamma^{-/-}\) mice were immunized orally with 25 mg of OVA in the absence of systemic challenge. Three days after oral immunization, T cells from spleen and Peyer’s patches were isolated and cultured in the same manner as described above. Culture supernatants were harvested after 2 days (for IL-2 and IFN-\(\gamma\)) and 4 days (for IL-4, IL-5, and IL-10) of incubation and analyzed by the respective cytokine-specific ELISA. Results are expressed as the mean \(\pm\) SE and show an example from three separate experiments containing five mice per group. *, \(p < 0.05\) vs PBS orally immunized group.](http://www.jimmunol.org/)

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responses (16, 18). On the other hand, IFN-γ−/− mice that were orally administered OVA showed higher levels of IL-4 (70–80 pg/ml), which favors the production of IgG1 and IgG2b subclasses, than did IFN-γ+/+ mice (5–10 pg/ml) (Fig. 2). Indeed, it has been shown that Ag-stimulated splenic CD4+ T cells from mice orally immunized with tetanus toxoid plus cholera toxin induce approximately 100 pg/ml of IL-4 in vitro, accounting for the subsequent induction of tetanus toxoid-specific IgG1 Ab responses (26). Among the several proposed explanations of how orally administered OVA elicits intact B cell responses in IFN-γ+/+ mice, we favor the one that suggests that IL-4 production by partially tolerized Th2 cells (as demonstrated by DTH and proliferative responses (Table III)) might contribute to the maintenance of B cell responses in these mice.

The role of IFN-γ in the induction of mucosally induced tolerance is further supported by recent studies in which spleen cells taken from mice given OVA by the oral route showed OVA-specific IFN-γ production when restimulated with Ag in vitro (32). In addition, a recent study has demonstrated that repeated oral administration of high doses of OVA to OVA TCR-transgenic mice results in IFN-γ-dominated immune responses in the Peyer’s patches (28). Though regulatory mechanisms for IFN-γ in the induction and maintenance of mucosally induced tolerance are not yet known, it is well established that IFN-γ contributes to the reduction of IL-4-producing Th2-type cells, which favor the production of IgG1 B cell responses (16, 18). Taken together, the immunologic consequences of systemic B cell tolerance induced by a high dose of oral Ag could be due to IFN-γ-mediated immune regulation, with significant suppression of Th2-type cells.

Oral administration of a high dose of OVA to IFN-γ−/− mice resulted in decreased T cell responses, though this reduced response was not significant, but B cell responses were intact. A state of unresponsiveness, or anergy, by immunocompetent cells has been reported within both T and B cell compartments (33). However, unresponsiveness of T cells with intact B cell responses has been reported in humans (34), and a recent study performed in transgenic mice showed that a small amount of autoantigen-induced T cell but not B cell tolerance (35). The B cell responses can be tolerized but generally require larger amounts of oral Ag (36, 37). Furthermore, the kinetics of tolerance induction are fundamentally different in T and B cells (38). For example, thymocytes become tolerant sooner and remain so longer than do bone marrow cells after injection of Ag, indicating that T cells are more easily tolerized and remain anergic longer than B cells. In this regard, it will be important to investigate the time course of Ag-specific Ab and T cell responses in both IFN-γ−/− and IFN-γ+/+ mice in comparison to IL-4−/− and IL-4+/+ mice following the induction of oral tolerance. Our group is currently conducting such experiments.

Since the level of TGF-β production had been considered to be the key factor for mucosally induced tolerance (10, 15, 39), it was important to examine levels of TGF-β following oral administration of OVA. Thus, TGF-β production in culture supernatants of Ag-stimulated splenic T cells and total TGF-β levels in the serum of orally immunized IFN-γ−/− and IFN-γ−/− mice were assessed by a sensitive ELISA. The levels of TGF-β were found to be similar in sera obtained from all groups tested. No detectable levels of TGF-β were seen in culture supernatants of OVA-stimulated splenic T cells isolated from IFN-γ−/− or IFN-γ−/− mice. These findings indicate that mucosally induced tolerance is not principally mediated by increased synthesis of TGF-β in our experimental system. However, others have suggested that TGF-β-producing CD4+ T cells (as a Th3 subset) may be essential for the induction of mucosally induced tolerance with low doses of Ag (15, 39). This suggestion was further supported by a recent study that demonstrated the presence of bystander suppression mediated by TGF-β following oral administration of low doses of Ag (40). These findings have indicated that the production of TGF-β is a necessary condition for the cytokine-mediated active suppression induced by low doses of oral Ag. On the other hand, a high dose tolerance was elicited by clonal anergy or deletion of Ag-specific cells (12, 13). Our present study has provided an additional possibility that IFN-γ production was maintained during the T cell unresponsiveness induced by a high oral dose of Ag, leading to inhibition of Th2 cells producing IL-4 with subsequent B cell unresponsiveness, especially for IgG1 subclass responses.

In summary, our study is the first to reveal that oral administration of a high dose of Ag induced brisk IFN-γ production, which may contribute to suppression of cytokine production (e.g., IL-4) by Th2-type cells, leading to the induction of B cell unresponsiveness. On the other hand, the partial maintenance of T cell responses including IL-4 production as well as intact B cell responses were elicited in mice with a disrupted IFN-γ gene, suggesting that IFN-γ is a key cytokine for induction and maintenance of mucosally induced tolerance.

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References

References


