Induction of IgG3 Secretion by Interferon γ: A Model for T Cell–independent Class Switching in Response to T Cell–independent Type 2 Antigens

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Summary

T cell–independent type 2 (TI-2), in contrast to T-dependent, antigens stimulate the production of murine IgG3. To investigate a possible role for cytokines in mediating the induction of this IgG subclass, we established an in vitro polyclonal model system for studying TI-2 antigen-mediated B cell activation by using dextran-conjugated anti-IgD antibody (αδ-dex). We demonstrate that interferon γ (IFN-γ) stimulates, and interleukin 4 inhibits, the expression of IgG3 by αδ-dex-activated cells. The production of IFN-γ by non-T cells in response to bacterial products, possibly capsular polysaccharides, may provide an explanation underlying the ability of TI antigens, which are unable to directly stimulate T cell–derived cytokines to induce Ig isotype switching.

Little is known regarding the parameters that regulate the humoral immune response to T cell–independent type 2 (TI-2)† antigens. Such antigens, exemplified by the haptenated polysaccharides, are typically abundant in bacterial cell walls. Immunization of mice with this class of antigen, in contrast to T cell–dependent antigens, stimulates a significant increase in serum levels of antigen-specific IgG3 (1). Since Ig class switching is widely viewed as a process that is regulated by the release of T cell–derived cytokines, the ability of TI-2 antigens to stimulate IgG3 production is especially intriguing. In this regard, it is of interest that no cytokine has been described that regulates its synthesis in a selective and positive manner.

The study of B cell responses to TI-2 antigens has been hampered by the low frequency of B cells specific for a given immunizing antigen. We recently described a model system in which resting murine mlgD+ B cells are polyclonally activated in vitro in a manner similar to the specific antibody response mediated by the prototypical TI-2 antigen, TNP-Ficoll (2, 3). Thus, like TNP-Ficoll, multiple anti-IgD mAb molecules covalently linked to a high molecular weight dextran backbone induce resting B cells to proliferate in the absence of significant Ig production. The addition of a differentiation factor, such as IL-5, stimulates the secretion of large amounts of polyclonal Ig by dextran-conjugated anti-IgD antibody (αδ-dex)-activated resting B cells as well as inducing much smaller amounts of antigen-specific Ig in response to TNP-Ficoll.

Materials and Methods

Mice. Female BALB/c mice were obtained from the National Institutes of Health Small Animals Division (Bethesda, MD) and were used at 7–10 wk of age.

Culture Medium. RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% FCS (Flow Laboratories, Inc., McLean, VA), L-glutamine (2 mM), 2-ME (0.05 mM), penicillin (50 μg/ml), and streptomycin (50 μg/ml) were used for culturing cells.

Reagents. αδ-dex conjugates were prepared as previously described (2). LPS W (Escherichia coli 0111:B4) (Difco Laboratories, Inc., Detroit, MI) was used at 20 μg/ml. Murine rIFN-γ was a kind gift of Genentech (South San Francisco, CA). Murine rIL-4 was a kind gift from Dr. Alan Levine (Monsanto Co., St. Louis, MO). Murine rIL-5 was a kind gift of Dr. Gregory Harriman (NIH). Affinity-purified polyclonal rabbit F(ab)'; anti-mouse IgG1 was prepared and tested for specificity (4). FITC-labeled monoclonal rat IgG1 anti-IgG3 was obtained from Pharmingen (San Diego, CA). PE-labeled, affinity-purified polyclonal goat anti-mouse IgM was purchased from Southern Biotechnology Associates (Birmingham, AL).

Preparation and Culture of B Cells. Enriched populations of small, resting splenic B cells were prepared as previously described and cultured at 1.25 × 10^6/ml.

Flow Cytometric Analysis and Cell Sorting. Fluorescence analyses and cell sorting were carried out on a FACSscan® and FACStar Plus® (Becton Dickinson & Co., Mountain View, CA), respectively, using...
logarithmic amplification. Cells obtained by cell sorting (mIgM−mIgG3−) were reanalyzed, immediately after their isolation, to determine their staining profile, and showed purities >99%.

**Measurement of Secreted Ig Isotype Concentrations by ELISA.** Ig isotype concentrations were measured by an ELISA as described (5).

Northern Blot Analysis. Total RNA, extracted from cultured cells, was separated, by electrophoresis, in a formaldehyde-containing 1% agarose gel and blotted onto a nylon membrane according to standard protocols. The blot was then sequentially hybridized with cDNA specific for Ig3 (6) and Ig1 (7) that were labeled with 32P deoxyctydine by the random hexamer method, and was subsequently exposed to x-ray film.

### Results

**IFN-γ Stimulates IgG3 Secretion by αδ-dex-activated B Cells.**

Upon testing a number of cytokines (IL-1, IL-2, IL-4, IL-6, TNF-α, IFN-α, and IFN-γ), we observed that IFN-γ stimulated IgG3 production by αδ-dex + IL-5-activated small, resting T-depleted spleen cells (B cell enriched). To study this further, log increments of rIFN-γ were added at the initiation of culture with αδ-dex, alone, or with αδ-dex + IL-5 (Fig. 1). IL-5 stimulated a large IgM secretory response by αδ-dex-activated B cells, with much smaller amounts of IgG3 and IgG1 (Fig. 1). Secreted levels of IgG2a (<1.2 ng/ml) (Fig. 1), as well as IgG2b (<2.4 ng/ml), IgA (<10 ng/ml), and IgE (<0.3 ng/ml), were below the limits of detection of the ELISA. Addition of IFN-γ at 1 and 10 U/ml to B cell cultures that were stimulated by αδ-dex + IL-5 induced a 6.3- and 17-fold enhancement in IgG3 secretion, respectively, over that seen with αδ-dex + IL-5, alone (Fig. 1). This induction was inhibited by the addition of anti-IFN-γ mAb (XMG-6) but not by an isotype-matched anti-NP control mAb (4-1) (data not shown). 100 U/ml of IFN-γ was typically inhibitory for both cellular proliferation as well as Ig production, and 0.1 U/ml of IFN-γ gave no measurable response (Fig. 1). Because even 10 U/ml of IFN-γ was found to be variably inhibitory for B cell proliferation and for overall Ig production, unless otherwise specified, we used 1 U/ml of IFN-γ in all subsequent experiments.

IFN-γ-mediated stimulation of IgG3 secretion by αδ-dex + IL-5-activated B cells was accompanied by a strong induction of IgG2a synthesis (Fig. 1), and a modest suppression in the production of IgM and IgG1 (Fig. 1). IFN-γ-mediated induction of IgG3 secretion by αδ-dex + IL-5-activated B cells was consistently observed in eight separate experiments. IFN-γ did not induce the secretion of IgG2b, IgE, or IgA (data not shown). In experiments performed in tandem, we confirmed our previous observations that IFN-γ also stimulates IgG2a production by LPS-activated B cells but inhibits the synthesis of IgG3 (8, and data not shown).

**IFN-γ Increases the Percentage of Cells Expressing mIgG3.** To determine whether IFN-γ also induced an increase in membrane (m)lgG3+ cells, we measured the percentage of cells expressing mIgG3 by flow cytometric analysis, 4 d after initiation of culture. B cells stimulated by αδ-dex + IL-5 in the presence of IFN-γ showed an over sixfold increase in the percentage of mIgG3+ cells compared with that seen in the presence of only αδ-dex + IL-5 (Fig. 2), whereas the percentage of mIgG1+ cells was essentially unchanged (data not shown). The IgG3 specificity of the antibody was ascertained as indicated (Fig. 2). Of interest, although IFN-γ alone did not induce an increase in the concentration of secreted IgM or IgG3 by αδ-dex-activated B cells, it stimulated a consistent approximately fourfold increase in the percentage of mIgG3+ cells over that seen with αδ-dex alone (Fig. 2). The percentage of mIgG3+ B cells induced by LPS vs. αδ-dex + IL-5 + IFN-γ was comparable (data not shown).

**IFN-γ Acts Directly on mIgM−mIgG3− B Cells to Stimulate IgG3 Secretion.** To determine whether IFN-γ directly stimulated B cells to synthesize IgG3 and to address whether it promoted this effect through induction of Ig class switching, we isolated, to high purity (>99%), resting, mIgM+mIgG3− spleen cells by cell sorting and cultured them in the presence of αδ-dex + IL-5 with or without IFN-γ. Similar to the...
Table 1. IFN-γ Acts Directly on MlgM⁺mlgG3⁻ B Cells to Stimulate IgG3 Production

| Ig secretion | IgM | IgG3 | IgG1 |
|--------------|-----|------|------|
| ng/ml        |     |      |      |
| mlgM⁺mlgG3⁻  |     |      |      |
| αδ-dex + IL-5| 15,625 | 94   | 375  |
| αδ-dex + IL-5 + IFN-γ | 10,000 | 850 | 33  |
| Unssorted    |     |      |      |
| αδ-dex + IL-5| 10,250 | 120  | 275  |
| αδ-dex + IL-5 + IFN-γ | 2,875  | 1,000 | 48  |

B cells were stained with FITC-anti-IgG3 + PE-anti-IgM. mlgM⁺mlgG3⁻ cells were isolated by cell sorting. Sorted cells were cultured at 1.25 × 10⁷/ml with αδ-dex (3 ng/ml) and rIL-5 (150 U/ml) with or without rIFN-γ (10 U/ml). Culture supernatants were removed 6 d after initiation of culture for measurement of IgM, IgG3, and IgG1 concentrations by ELISA.

IFN-γ Selectively Stimulates Steady-State Levels of Germline γ3 RNA. An increase in the steady-state levels of germline C₃ RNA specific for a particular C₃ gene typically precedes class switching to the expression of that gene. To further assess whether IFN-γ acts as a switch factor for IgG3, we determined whether it selectively induced germline γ3 RNA in αδ-dex + IL-5-activated B cells. B cells were stimulated for 2 d with αδ-dex ± IL-5 ± IFN-γ, and Northern blot analysis was performed using cDNA specific for germline γ3 (Iγ3) (6) or γ1 (Iγ1) (7) RNA. IFN-γ stimulated a significant increase in the results obtained with unsorted resting B cells, IFN-γ stimulated an approximately ninefold increase in the secretion of IgG3 while inhibiting the production of IgM and IgG1 (Table 1).

Figure 2. IFN-γ increases the percentage of membrane mlgG3⁺ cells in αδ-dex + IL-5-containing B cell cultures. B cells were stimulated with αδ-dex (3 ng/ml) ± rIL-5 (150 U/ml) ± rIFN-γ (1 U/ml). Cultured cells were removed on day 4 for quantitation of mlgG3⁺ B cells by flow cytometric analysis. 15,000 cells were analyzed from each group. Essentially identical results were obtained in a second experiment.

Figure 3. IFN-γ induces germline γ3, but not γ1, RNA. B cells were stimulated for 2 d with αδ-dex (3 ng/ml) ± IL-5 (150 U/ml) ± IFN-γ (1 U/ml). Total RNA was extracted and Northern blot analysis was performed using the indicated probes to detect C₃-specific germline RNA. Ethidium bromide staining of the Northern blot indicates equal transfer of RNA. Similar results were obtained in two additional experiments.
**Table 2. IL-4 Inhibits IFN-γ-mediated IgG3 Production**

| Ig secretion | IgM | IgG3 | IgG2a | IgG1 |
|--------------|-----|------|-------|------|
| **ng/ml**    |     |      |       |      |
| αδ-dex + IL-5 | 9,250 | 38 | 22 | 300 |
| αδ-dex + IL-5 + IFN-γ | 7,000 | 255 | 335 | 330 |
| αδ-dex + IL-5 + IFN-γ + IL-4 | 30,000 | 37 | 30 | 14,400 |

B cells were stimulated with αδ-dex (3 ng/ml) ± rIL-5 (150 U/ml) ± rIFN-γ (1 U/ml) ± rIL-4 (1,000 U/ml) as indicated. Supernatants were removed 6 d after initiation of culture for measurement of Ig isotype concentrations by ELISA. The data are representative of three experiments.

IL-4 Inhibits IFN-γ-mediated Induction of IgG3. We previously demonstrated that IL-4 inhibits IFN-γ-mediated IgG2a production by B cells (8). The data in this manuscript demonstrate that IL-4 also strongly inhibited IFN-γ-mediated IgG3, as well as IgG2a production, by αδ-dex + IL-5-activated B cells (Table 2). Maximal inhibition was observed at 100 U/ml of IL-4, with only modest suppression at 10 U/ml (data not shown). This IL-4-mediated inhibition of IgG3 secretion is accompanied by a complete abrogation in the induction of mIgG3+ cells (data not shown). By contrast, IL-4 induced a striking increase in IgG1 secretion and in the percentage of mIgG1+ cells that are activated with αδ-dex + IL-5 + IFN-γ, as well as further increasing the secretion of IgM (Table 2, data not shown). Maximal stimulation of IgM and IgG1 secretion was observed at 1,000 U/ml of IL4, with only modest stimulation seen at 100 U/ml (data not shown). IL-4 was previously shown to stimulate IgG1 secretion by B cells activated by αδ-dex + IL-5 (data not shown).

Discussion

We favor the view that IFN-γ stimulates IgG3 secretion by promoting the class switch to IgG3. IFN-γ acts directly on αδ-dex-activated mIgM+ mIgG3+ B cells to stimulate IgG3 secretion; IFN-γ is selective in its response, stimulating the production of only IgG3 and IgG2a while suppressing or leaving unaffected the synthesis of all other Ig isotypes.

IL-4 Inhibits IFN-γ-mediated Induction of IgG3 is dependent upon the mode of B cell activation, since IFN-γ inhibits IgG3 production by LPS-activated B cells (8). Of interest, in this context, is our recent observation that B cells stimulated by αδ-dex + IL-5, in contrast to cells induced by LPS or T cells, fail to secrete IgE in the presence of IL-4 (10). Thus, the presence of certain cytokines during an immune response may be necessary, but not sufficient, for determining the profile of secreted Ig isotypes that is elicited.

The mechanism by which TI-2 antigens elicit an IgG3 response in vivo, in the apparent absence of T cells, is unknown. The ability of IFN-γ to stimulate IgG3 production by B cells activated by αδ-dex, a TI-2-like antigen, suggests a physiologic role for this cytokine in IgG3 regulation. In this regard, Wherry et al. (11) have demonstrated that infection with the bacterium *Listeria monocytogenes* stimulates IFN-γ production by NK cells in a T-independent fashion. More recently, work by Van Den Eertwagh et al. (12) indicates that immunization of mice with the TI-2 antigen, TNP-Ficoll, induces IFN-γ production in vivo, in part through synthesis by NK cells. Finally, Amigorena et al. (13) have shown that NK cells can stimulate IgG2a production by LPS-activated B cells in vitro by releasing IFN-γ in response to IL2. Thus, the synthesis of IFN-γ by non-T cells during certain types of immunization could provide a means for stimulating Ig class switching in response to TI antigens.
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