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

By Clifford M. Snapper,* Tina M. McIntyre,* Raya Mandler,* Ligia M. T. Pecanha,‡ F. D. Finkelman,‡ Andrew Lees,‡ and James J. Mond‡

From the Departments of *Pathology and ‡Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

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)[1] 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 mIgD+ 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 was a kind gift of Dr. Gregory Harriman (NIH). Affinity-purified polyclonal rabbit F(ab')₂ anti-mouse IgG1 was prepared and tested for specificity (4). FITC-labeled monoclonal rat IgG1 anti-mouse 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 × 10⁵/ml.

Flow Cytometric Analysis and Cell Sorting. Fluorescence analyses and cell sorting were carried out on a FACScan® and FACStar Plus® (Becton Dickinson & Co., Mountain View, CA), respectively, using

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[1] *Abbreviations used in this paper:* αδ-dex, dextran-conjugated anti-IgD antibody; TI-2, T cell-independent type 2.

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 Iγ3 (6) and Iγ1 (7) that were labeled with ^{32}P-deoxycytidine by the random hexamer method, and was subsequently exposed to x-ray film.

Results

IFN-γ Stimulates IgG3 Secretion by $\alpha\delta$-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 $\alpha\delta$-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 $\alpha\delta$-dex, alone, or with $\alpha\delta$-dex + IL-5 (Fig. 1). IL-5 stimulated a large IgM secretory response by $\alpha\delta$-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 $\alpha\delta$-dex + IL-5 induced a 6.3- and 17-fold enhancement in IgG3 secretion, respectively, over that seen with $\alpha\delta$-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 (J4-1) (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 produc-

tion, unless otherwise specified, we used 1 U/ml of IFN-γ in all subsequent experiments.

IFN-γ-mediated stimulation of IgG3 secretion by $\alpha\delta$-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 $\alpha\delta$-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)IgG3$^+$ cells, we measured the percentage of cells expressing mIgG3 by flow cytometric analysis, 4 d after initiation of culture. B cells stimulated by $\alpha\delta$-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 $\alpha\delta$-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 $\alpha\delta$-dex-activated B cells, it stimulated a consistent approximately fourfold increase in the percentage of mIgG3$^+$ cells over that seen with $\alpha\delta$-dex alone (Fig. 2). The percentage of mIgG3$^+$ B cells induced by LPS vs. $\alpha\delta$-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 $\alpha\delta$-dex + IL-5 with or without IFN-γ. Similar to the
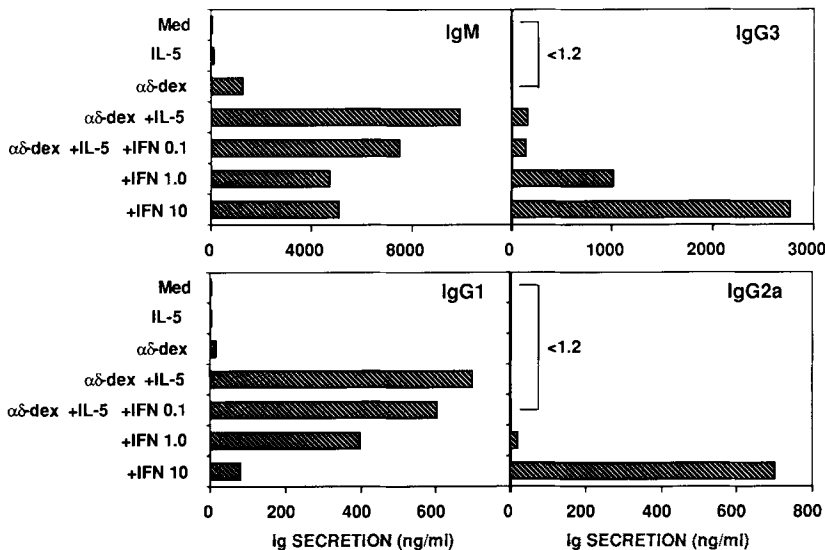
Figure 1. IFN-γ selectively stimulates IgG3 and IgG2a secretion by $\alpha\delta$-dex + IL-5–activated resting B cells. B cells were stimulated with $\alpha\delta$-dex (3 ng/ml), rIL-5 (150 U/ml), and/or log increments of rIFN-γ as indicated. Cultures were carried for 6 d, after which culture supernatants were removed for analysis of Ig isotypes by ELISA. Time course experiments indicated that maximum concentrations of Ig were obtained by 6 d after initiation of culture (data not shown). Cultures were established in triplicate for each point. The data are representative of eight experiments.
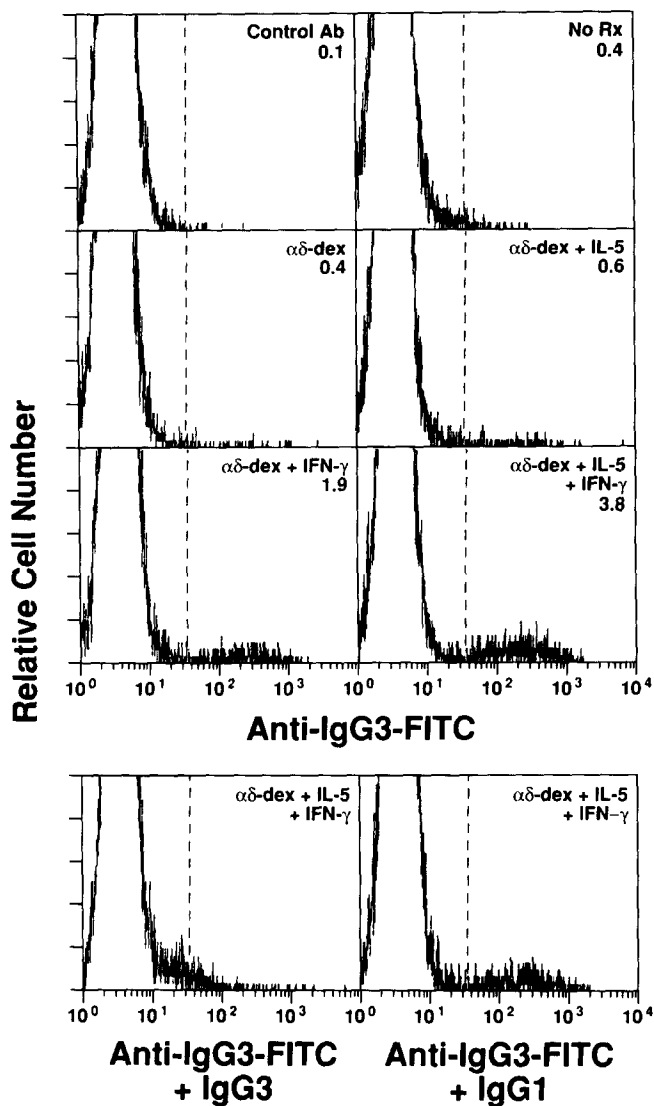
Figure 2. IFN-γ increases the percentage of membrane (m)IgG3⁺ cells in αδ-dex + IL-5–containing B cell cultures. B cells were stimulated with αδ-dex (3 ng/ml) ± rIL-5 (150 U/ml) ± rIFN-γ (1 U/ml). Cultured cells were removed on day 4 for quantitation of mIgG3⁺ B cells by flow cytofluorometric analysis. 15,000 cells were analyzed from each group. Essentially identical results were obtained in a second experiment.
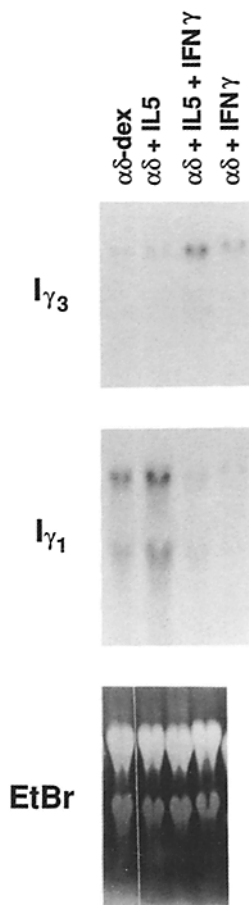
Table 1. *IFN-γ Acts Directly on MIgM⁺mIgG3⁻ B Cells to Stimulate IgG3 Production*

	Ig secretion		
	IgM	IgG3	IgG1
	ng/ml		
mIgM⁺mIgG3⁻			
αδ-dex + IL-5	15,625	94	375
αδ-dex + IL-5 + IFN-γ	10,000	850	33
Unsorted			
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. mIgM⁺mIgG3⁻ cells were isolated by cell sorting. Sorted cells were cultured at 1.25 × 10⁵/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.

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).

IFN-γ Selectively Stimulates Steady-State Levels of Germline γ3 RNA. An increase in the steady-state levels of germline C_H RNA specific for a particular C_H 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 in-

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 C_H-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.

crease in the steady-state level of γ3, but inhibited γ1 germline RNA (Fig. 3) by B cells stimulated with αδ-dex or αδ-dex + IL-5. IFN-γ induction of germline γ3 transcripts by αδ-dex-activated cells was greater in the presence of IL-5, despite the fact that IL-5 itself had no effect on the level of γ3 germline transcripts by αδ-dex-activated cells.

IL-4 Inhibits IFN-γ-mediated Induction of IgG3. We previously demonstrated that IL-4 inhibits IFN-γ-mediated IgG2a production by LPS-activated 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 IL-4, with only modest stimulation seen at 100 U/ml (data not shown). IL-4 was previously shown to stimulate IgG1 secretion by LPS-activated B cells (9). Experiments performed

in tandem indicate that IL-4 induces even higher levels of IgG1 by B cells activated by αδ-dex + IL-5, in the absence of IFN-γ (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. In addition, IFN-γ increases the percentage of mIgG3⁺ cells induced by αδ-dex + IL-5 without affecting the percentage of cells bearing mIgG1. Finally, IFN-γ increases the steady-state levels of germline γ3, but not γ1, RNA by αδ-dex + IL-5-activated B cells.

The 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 IL-2. 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.

References

1. Perlmutter, R.M., D. Hansburg, D.E. Briles, R.A. Nicolotti, and J.M. Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol.* 121:566.
2. Brunswick, M., F.D. Finkelman, P.F. Highet, J.K. Inman, H.M. Dintzis, and J.J. Mond. 1988. Picogram quantities of anti-Ig antibodies coupled to dextran induce B cell proliferation. *J. Immunol.* 140:3364.
3. Pecanha, L.M.T., C.M. Snapper, F.D. Finkelman, and J.J. Mond. 1991. Dextran-conjugated anti-Ig antibodies as a model for T cell-independent type 2 antigen-mediated stimulation of Ig secretion in vitro. I. lymphokine dependence. *J. Immunol.* 146:833.
4. Finkelman, F.D., I. Scher, J.J. Mond, S. Kessler, J.T. Kung, and E.S. Metcalf. 1982. Polyclonal activation of the murine immune system by an antibody to IgD. II. Generation of polyclonal antibody production and cells with surface IgG. *J. Immunol.* 129:638.
5. Snapper, C.M., and W.E. Paul. 1987. B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial lipolysaccharide. *J. Immunol.* 139:10.
6. Rothman, P., S. Lutzker, B. Gorham, V. Stewart, R. Coffman, and F.W. Alt. 1990. Structure and expression of germline immunoglobulin g3 heavy chain gene transcripts: implications for mitogen and lymphokine directed class-switching. *Int. Immunol.* 2:621.
7. Mowatt, M.R., and W.A. Dunnick. 1986. DNA sequence of the murine γ1 switch segment reveals novel structural elements. *J. Immunol.* 136:2674.
8. Snapper, C.M., and W.E. Paul. 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science (Wash. DC).* 236:944.
9. Isakson, P.C., E. Pure, E.S. Vitetta, and P.H. Krammer. 1982. T cell-derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* 155:734.
10. Snapper, C.M., L.M.T. Pecanha, A.D. Levine, and J.J. Mond. 1991. IgE class switching is critically dependent upon the nature of the B cell activator, in addition to the presence of IL-4. *J. Immunol.* 147:1163.
11. Wherry, J.C., R.D. Schreiber, and E.R. Unanue. 1991. Regulation of gamma interferon production by natural killer cells in scid mice: roles of tumor necrosis factor and bacterial stimuli. *Infect. Immun.* 59:1709.
12. Van Den Eertwegh, A.J.M., M.J. Fasbender, M.M. Schellekens, A.V. Oudenaren, W.J.A. Boersma, and E. Claassen. 1991. In vivo kinetics and characterization of IFN-γ-producing cells during a thymus-independent immune response. *J. Immunol.* 147:439.
13. Amigorena, S., C. Bonnerot, W.H. Fridman, and J.-L. Teillaud. 1990. Recombinant interleukin 2-activated natural killer cells regulate IgG2a production. *Eur. J. Immunol.* 20:1781.