Regulation by Interferon α of Immunoglobulin Isotype Selection and Lymphokine Production in Mice

By Fred D. Finkelman,* Antonela Svetic,‡ Ion Gresser,¶ Clifford Snapper,§ Joanne Holmes,* Paul P. Trotta,** Ildy M. Katona,*‖ and William C. Gause‡

*From the Departments of *Medicine, ‡Microbiology, §Pathology, and ‖Pediatrics of the Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; the ¶Laboratory of Viral Oncology, UTR 274 CNRS, Groupe de Lab. I.R.S.C.-BP8, 94801 Villejuif Cedex, France; and the **Department of Biotechnology-Biochemistry, Schering-Plough Research, Bloomfield, New Jersey 07003*

Summary

Antigens and infectious agents that stimulate interferon α(IFN-α) production in mice induce antibody responses that are predominantly of the immunoglobulin (Ig)G2a isotype and contain little or no IgE. This suggested the possibility that IFN-α might have a role in directing Ig isotype selection. Consistent with this possibility, we have found that injection of mice with recombinant mouse IFN-α suppresses IgE secretion, enhances IgG2a secretion, and has no independent effect on IgG1 secretion in mice stimulated with a foreign anti-IgD antibody. Injection of mice with polyinosinic acid·polycytidylic acid (poly I·C), an inducer of macrophage IFN-α production, also suppresses the anti-IgD antibody-induced IgE response and stimulates the IgG2a response; these effects are blocked by a sheep antibody that neutralizes mouse IFN-α/β. Both recombinant IFN-α and poly I·C have maximum IgE suppressive and IgG2a stimulatory effects when injected early in the anti-IgD antibody-induced immune response. Addition of IFN-α to mouse B cells cultured with lipopolysaccharide (LPS) + interleukin 4(IL-4) suppresses both IgG1 and IgE production, but much less potently than IFN-γ. IFN-α suppresses anti-IgD antibody-induced increases in the level of splenic IL-4 mRNA, but enhances the anti-IgD antibody-induced increase in the splenic level of IFN-γ mRNA. These results are consistent with the effect of IFN-α on Ig isotype expression in mice, as IL-4 stimulates IgE and suppresses IgG2a secretion while IFN-γ exerts opposite effects. These observations suggest that antigen presenting cells, by secreting IFN-α early in the course of an immune response, can influence the nature of that response both through direct effects on B cells and by influencing the differentiation of T cells.

Differential production of cytokines during an immune response has important regulatory effects on the nature of that response. Responses in mice that are characterized by substantial IL-4 but little IFN-γ production generate IgG1- and IgE-secreting cells, with little IgG2a (1, 2). Responses in which the production of IFN-γ predominates are characterized by IgG2a production, activation of macrophages to kill intracellular parasites, delayed type hypersensitivity, and little or no IgE production (2–7).

The correlations between cytokine production and selection of Ig isotypes in large part reflect direct effects of IL-4 and IFN-γ on B lymphocytes. In the presence of bacterial LPS, IL-4 stimulates purified B cells to secrete IgG1 and IgE, and suppresses IgG2a production, while IFN-γ has the opposite effect (8–11). Furthermore, anti–IL-4 antibody blocks IgE responses and can enhance IgG2a responses in vivo (12, 13). IFN-γ has the same effects and also inhibits production in vivo of IgG1, and anti–IFN-γ antibody can enhance production in vivo of IgG1 and IgE and inhibit the production of IgG2a (7).

Cytokines, however, may also have indirect effects on Ig isotype selection. Mouse CD4$^+$ T cell clones prepared after longterm immune stimulation tend to produce exclusively either of two sets of cytokines. Th1 cells secrete IL-2, IFN-γ, and lymphotoxin, but not IL-4, IL-5, IL-6, or IL-10, while Th2 cells have the opposite cytokine secretory profile (3, 4, 14). IFN-γ inhibits the proliferation of Th2, but not Th1 cells (15), while IL-10 inhibits cytokine secretion by Th1, but not Th2 cells (16). Thus, once one CD4$^+$ T cell subset becomes dominant during an immune response, a mechanism

exists to maintain that dominance. The mechanisms by which resting CD4$^+$ T cells are induced during activation to differentiate into cells that secrete predominantly Th1 or Th2 cytokines are less clear. One possibility is that non-T cells might be stimulated early in an immune response to produce factors that can influence T cell differentiation.

IFN-α is an attractive candidate for a cytokine that might influence the characteristics of an immune response both through direct actions on effector cells and through the regulation of T cell differentiation. Large quantities of IFN-α are produced early in some immune responses by macrophages (17). Thus, macrophages might simultaneously present antigen to T cells, activating them, and influence their pathway of differentiation by secreting IFN-α. Second, IFN-α, like IFN-γ, inhibits IgE production by human PBCs that are cultured with IL-4 (18). Third, the injection of mice with killed, fixed, *Brucella abortus* (BA),[1] an agent known to stimulate the production of both IFN-γ and IFN-α (7, 19, 20), blocks the generation of both IgG1 and IgE responses in vivo (7). Administration of anti-IFN-γ antibody to these mice blocks the inhibitory effect of BA on IgG1 production, but fails to block its inhibitory effect on IgE production (7). We hypothesized that the production of IFN-α in response to BA might be responsible for this IFN-γ-independent effect.

To investigate the possible role of IFN-α in the regulation of Ig isotype secretion we have studied the effects of recombinant IFN-α, agents that induce endogenous IFN-α production, and a polyclonal anti-IFN-α/β antibody on the Ig isotypes secreted in mice that have been stimulated to make a large polyclonal Ig response by the injection of anti-IgD antibodies. These studies provide evidence that IFN-α produced early in the course of an immune response inhibits IgE production and stimulates IgG2a production, and that these effects may result from a decrease in T cell IL-4 gene expression as well as from a direct effect on the B cell.

Materials and Methods

Animals. BALB/c female mice were purchased from the Small Animals Division of the National Cancer Institute, NIH, and were used at 8 to 12 wk-of-age. The experiments herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare Publication (National Institutes of Health) 78-23.

Antibodies. The following antibodies were prepared as described: affinity purified goat anti-mouse IgD (GaMδ) (21); FF1-4D5 (a mouse IgG2a mAb of the *b* allotype specific for IgD of the *a* allotype) (22); Hδ^a 1 (a mouse IgG2b mAb of the *b* allotype specific for IgD of the *a* allotype) (23); XMG-6 (a rat IgG1 that neutralizes mouse IFN-γ) (24); J4-1 (a rat IgG1 anti-NP mAb, used as a control) (23); 24G2 (a rat IgG2b antibody specific for the mouse FcγRII) (25); a polyclonal sheep antibody that neutralizes mouse IFN-α and IFN-β (SaIFN-α/β) (26); and normal sheep serum.

Immune Reagents. Recombinant murine IFN-α, generously provided by Schering-Plough Research (Bloomfield, NJ), was diluted in 0.5 M NaCl/0.02 M Tris, pH 7.4, and kept frozen at $-70°C$ until used. Different lots had 0.85–1.7 $\times$ 10^7 IU/mg of antiviral activity. All lots were free of detectable LPS by the limulus assay. Recombinant mouse IL-4 (a gift of Dr. William E. Paul, Bethesda, MD) was produced in the baculovirus system and purified by passage over an anti-IL-4 (11B11) (27) affinity column, followed by further purification by HPLC. IL-4 was quantitated with a CT.4S proliferation assay (28). Polyinosinic acid·polycytidylic acid (poly I·C) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Lipopolysaccharide (LPS W, extracted from *Escherichia coli* 0111:B4) was purchased from Difco Laboratories, Inc. (Detroit, MI).

Cell Cultures. Enriched populations of B cells were obtained from BALB/c spleens by treatment with anti-Thy 1 (mAb 30H12) (29) plus complement. Small dense B cells were obtained by discontinuous Percoll gradient centrifugation. Cells that formed a band between 60% and 70% Percoll and had a density of 1.081–1.086 g/ml were considered to be resting B cells. Cells were cultured for 6 d at 1.25 $\times$ 10^5 cells/ml in 6-well flat-bottom Costar plates (Costar, Cambridge, MA) in medium RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-ME, 50 μg/ml penicillin, and 50 μg/ml streptomycin. LPS was added to some cultures at 20 μg/ml, IL-4 was added to some cultures at 10,000 U/ml, and anti-IFN-γ mAb (XMG-6) was added to some cultures at 5 μg/ml.

Quantitation of Ig Isotypes. Serum IgG1 and IgG2a were quantitated by radial immunodiffusion, using rabbit anti-mouse IgG1 antiserum (30) and a sheep anti-mouse IgG2a antiserum purchased from The Binding Site, Ltd. (Birmingham, UK). IgG1 and IgG2a in culture supernatants, and IgE in serum and in culture supernatants, were quantitated by ELISA (31).

Quantitation of Cytokine mRNA. A reverse transcriptase-PCR approach (32) was used to determine relative quantities of mRNA for IL-4 and IFN-γ (details below). This approach has been shown to reproducibly detect less than 1.3-fold differences in mRNA content.

Isolation and Purification of RNA. RNase-free plastic and water were used throughout the assay. Tissues were homogenized in RNAzol (Cinna/Biotecx, Friendswood, TX) at 50 mg of spleen per ml. 0.2 ml of chloroform was added per 2 ml of homogenate, the samples were tightly covered, shaken vigorously for 15 s, and then incubated on ice for 15 min. The suspension was centrifuged at 12,000 *g* (4°C) for 15 min. The aqueous phase was transferred to a fresh tube, to which an equal volume of isopropanol was added. After mixing, the samples were incubated for 45 min at $-20°C$. Samples were centrifuged for 15 min at 12,000 *g* (4°C). The resultant RNA precipitate was transferred to a 1.2 ml centrifuge tube and washed twice with 75% ethanol. The final preparation was suspended in water and quantitated spectrophotometrically.

To ascertain whether the RNA was intact and whether the concentration had been determined correctly, the purified RNA (10 μg) was electrophoresed on a 2% formaldehyde gel containing ethidium bromide (33). The gel was photographed and individual lanes were examined for the presence of the 18S and 28S ribosomal bands, the absence of RNA degradation, and whether equal loading of RNA on each lane had occurred.

Reverse Transcriptase Reaction. The coupled reverse transcription/polymerase chain reaction used here was a modification of that described by Diamond et al. (34). Reverse transcription of RNA was performed in a 25 μl final volume containing: (*a*) 2.5 μl of a 10 mM mix of all four deoxynucleotide triphosphates (dNTP);

[1] *Abbreviations used this paper:* BA, *Brucella abortus*; GaMδ, affinity-purified goat antibody specific for IgD; HPRT, hypoxanthine-guanine phosphoribosyl transferase; MaMδ, a combination of two mouse alloantibodies specific for mouse IgD; poly I·C, polyinosinic acid·polycytidylic acid.

(*b*) 5 μl of 5× reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$; (*c*) 2 μl 0.1 M DTT; (*d*) 2 μl random hexamers (0.5 U/25 μl); (*e*) 3 μl total RNA (1 μg/μl); (*f*) 8.7 μl double distilled H_2O. This was mixed, heated to 70°C for 5 min to denature the RNA, cooled on ice and 1.2 μl of reverse transcriptase (RT) (200 U/ml) was added. This was again mixed, centrifuged to pellet any precipitate, and incubated at 37°C for 60 min. The reaction was next heated at 90°C for 5 min, then quickly cooled on ice.

Polymerase Chain Reaction. The primers for amplification during the PCR have been described (32). Product amplified from cDNA could be distinguished from any amplified from genomic DNA, because the primers amplified genomic DNA introns as well as exons. To the 12 μl of RT mix the following components were added: (*a*) 4 μl dNTP mix (10 mM); (*b*) 5 μl 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$, 1 mg/ml BSA); (*c*) 5 μl sense primer (0.2 μg/μl); (*d*) 2 μl anti-sense primer (0.2 μg/ml); (*e*) 24 μl double distilled H_2O; (*f*) 0.5 μl Taq polymerase (5 U/ml). After an initial incubation at 95°C for 5 min, temperature cycling was initiated with each cycle as follows: (*a*) 95°C for 1 min (denaturation); (*b*) 53–55°C (depending on the cytokine to be amplified) for 1 min (annealing of primers); (*c*) 72°C for 3 min (primer extension). For each gene product, the optimum number of cycles was determined experimentally, and was defined as that number of cycles that would achieve a detectable concentration which was well below saturating conditions.

To verify that equal amounts of RNA were added in each PCR reaction within an experiment and to verify a uniform amplification process, hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA was also reverse transcribed and amplified for each assay (32).

Detection of the Amplified Product by Southern Blot Analysis. 10 μl of the final reaction mix was run on a 1% agarose gel at 120 volts for 3 h. The gel was then denatured by soaking for 45 min in several volumes of 1.5 M NaCl, 0.5 N NaOH with constant, gentle agitation. The gel was then rinsed briefly in deionized water, and neutralized by soaking for 45 min in several volumes of 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant agitation.

The DNA was then transferred to a nylon membrane by standard blotting procedures (35). The membrane was UV crosslinked using the UV Stratalinker 1800 (Stratagene, La Jolla, CA). Blots were prehybridized at 42°C for 6 h in a solution containing 6× SSPE and 1% SDS. The probes were specifically selected to hybridize to a portion of the amplified segment between the nucleotide sequences complementary to the primers. This ensured the identity of the segment amplified. After hybridization, blots were washed for 15 min in 6× SSPE, 0.1% SDS and then 3–4 min in 2× or 1× SSPE at 49°C. Autoradiographs were exposed at −70°C using Kodak XAR-2 film.

Video Densitometry. Following autoradiography, the resultant bands on the XAR-2 film represent areas of specific probe hybridization. The band intensity was quantitated using one of two methods: (*a*) With the JAVA video densitometry system (Jandel Scientific, Corte Madera, CA), several autoradiographic exposures were taken of each blot to ensure that the signal from each band was in the linear range of the film response. (*b*) Blots were exposed to a phosphor screen that was then read directly with a 400 series phosphoimager (Molecular Dynamics, Sunnyvale, CA). The high sensitivity and broad dynamic range of the phosphoimager facilitated quantitation.

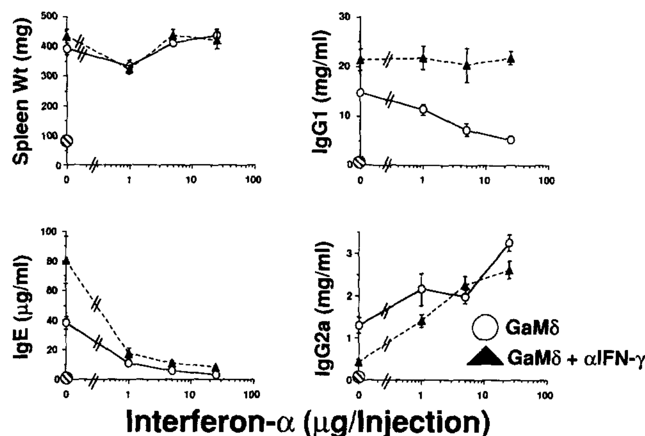

Figure 1. Effect of IFN-α on the increases in spleen weight and serum Ig levels in GaMδ-injected mice. BALB/c mice (5/group) were injected i.v. with GaMδ (800 μg) or with GaMδ plus anti–IFN-γ mAb (1 mg). Mice were injected i.p. with saline or the doses of recombinant mouse IFN-α shown twice daily 2, 3, and 4 d after GaMδ injection. Mice were bled and sacrificed 8 d after GaMδ injection. Spleens were weighed, serum IgG1 and IgG2a levels were determined by radial immunodiffusion, and serum IgE levels were determined by ELISA. Data are shown as geometric means and standard errors. Crosshatched circles show values observed in untreated mice.

Results

Effect of IFN-α on Increases in Serum Immunoglobulin (Ig) Levels and Spleen Weight in GaMδ-injected Mice. To determine the effect of recombinant mouse IFN-α on the humoral immune response to GaMδ, mice were injected i.v. with 800 μg of GaMδ and 2, 3, and 4 d later were injected twice daily i.p. with 0.2 ml of 0.5 M NaCl/0.02 M Tris, pH 7.4, or with the same buffer containing 1, 5, or 25 μg of rIFN-α. Analysis of sera obtained 8 d after GaMδ injection indicated that GaMδ stimulated an approximately 15-fold increase in serum IgG2a and a greater than 100-fold increases in serum IgG1 and IgE levels (Fig. 1). In addition, an approximately five-fold increase in spleen weight was observed in GaMδ-injected mice. IFN-α, at the highest dose tested, suppressed serum IgE levels by greater than 90% and IgG1 levels by approximately 60%, while enhancing serum IgG2a levels two–three-fold, but had no effect on spleen weight. To determine if any of these effects were dependent upon the presence of IFN-γ, additional groups of mice that were injected with both GaMδ and 1 mg of anti–IFN-γ mAb were similarly treated with buffer or IFN-α. In the absence of IFN-α, anti–IFN-γ enhanced the GaMδ-induced IgG1 and IgE responses and suppressed the GaMδ-induced IgG2a response. IFN-α strongly suppressed the IgE response and stimulated the IgG2a response in these mice, but had no effect on the IgG1 response. Thus, IFN-α, independently of IFN-γ, can inhibit IgE production and enhance IgG2a production, but has no independent effect on the IgG1 response. The effects of IFN-α on Ig isotype secretion were not simply the result of altered kinetics of Ig secretion. When administered on days two through four after GaMδ injection, IFN-α enhanced serum IgG2a levels and inhibited serum IgE levels both 8 and 10 d after GaMδ treatment (Fig. 2). However, a large

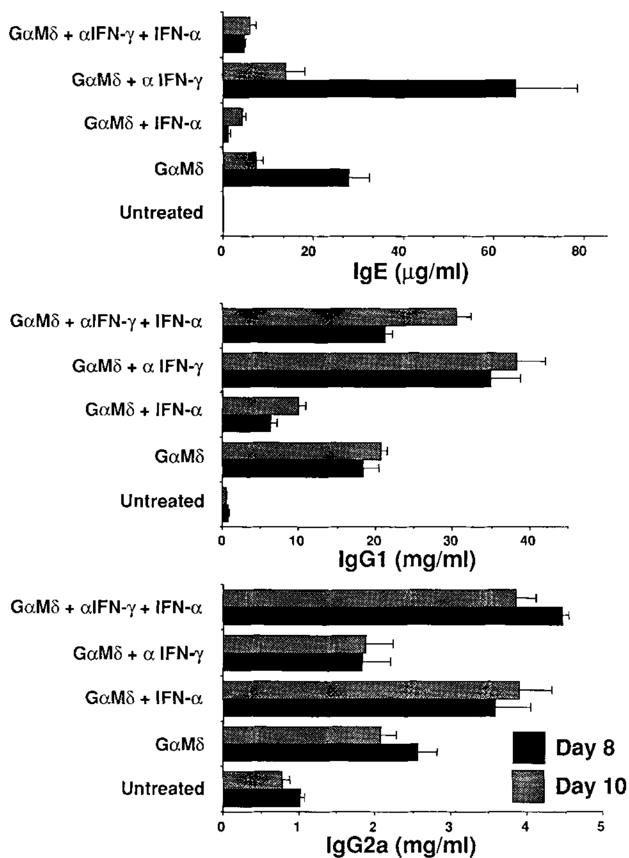

Figure 2. Effect of IFN-α on serum Ig levels in GaMδ-injected mice. BALB/c mice were treated with GaMδ ± anti-IFN-γ ± IFN-α as in the legend to Fig. 1, and were bled 8 and 10 d after GaMδ injection. Geometric means and standard errors of serum Ig levels are shown.

day 8 to day 10 decrease in serum IgE levels was seen in GaMδ-treated mice, while IgE levels remained at a low, but stable level in mice that also received IFN-α.

IFN-α Has an Early Suppressive Effect on the Generation of an IgE Response in GaMδ-injected Mice. Previous studies have demonstrated that a 3-d course of IFN-γ has its greatest suppressive effect on IgE secretion by GaMδ-injected mice if administered 2 through 4 d after GaMδ injection (7). To determine if IFN-α suppression of the IgE response followed the same kinetic pattern, GaMδ-treated mice were injected twice daily with IFN-α on the day of and on the 2 d following GaMδ injection, or on days 2 through 4 after GaMδ. In this experiment, a suboptimal dose of IFN-α was used (10 μg twice a d), so that minor time-related differences in IFN-α suppression of IgE production could be optimally detected, and all mice were treated with anti-IFN-γ mAb, to eliminate any effects of this cytokine on the IgE response. Sera were obtained 8 d after GaMδ injection and serum Ig levels were measured. Treatment with IFN-α, on the day of and on the 2 d following GaMδ injection, or on days 2 through 4 after GaMδ, decreased serum IgE levels by a factor of approximately three, and had no significant effect on IgG1 levels (data not shown).

Endogenous IFN-α/β Production Suppresses IgE Secretion. To investigate the possibility that endogenously produced IFN-α or IFN-β could effect Ig isotype selection in the same way as injected recombinant IFN-α, we studied the effects of injecting poly I·C, a potent inducer of endogenous IFN-α/β production (36-38), on the immune responses induced by GaMδ. Mice in this experiment also received anti-IFN-γ mAb, because we have found that poly I·C can induce the production of IFN-γ as well as IFN-α/β (Snapper, C.M., H. Yamaguchi, J.F. Urban, Jr., and F.D. Finkelman, manuscript submitted for publication). Injection of a single dose of poly I·C (100 μg i.p.) on the day of or 1 or 2 d after GaMδ suppressed the GaMδ-induced IgE response by approximately 90%, but had no suppressive effect on serum IgG1 levels (data not shown).

Since poly I·C has important biological effects in addition to the induction of IFN production, it was possible that the effects of poly I·C on Ig isotype regulation were unrelated to its stimulation of an IFN-α response. To determine the relationship between the poly I·C effects and IFN-α (or IFN-β) production, we studied the ability of a sheep anti-IFN-α/β antiserum to block the effects of poly I·C on Ig isotype selection in anti-IgD antibody-treated mice. In these experiments, a pair of mouse IgG2 antibodies of the *b* allotype that are specific for IgD of the *a* allotype (Hδ^a/1 and FF1-4D5, abbreviated as MaMδ) were used instead of GaMδ. Because poly I·C rapidly stimulates macrophage production of IFN-α, mice were injected with anti-IFN-α/β antiserum and anti-IFN-γ mAb 1 d before their injection with the anti-δ mAbs and poly I·C. In mice pre-treated with anti-IFN-γ mAb plus a control sheep serum, a 200 μg dose of poly I·C inhibited the IgE response induced by MaMδ by greater than 90% (Fig. 3). No inhibition of the IgE response by poly I·C was observed, however, when mice were pretreated with sheep anti-IFN-α/β rather than with the control sheep serum. In contrast, serum IgG2a levels were approximately twice as high in mice treated with MaMδ, poly I·C, and anti-IFN-γ mAb in the presence of control sheep serum as in mice injected with the same agents in the presence of sheep anti-IFN-α/β antiserum (Fig. 3). As expected, neither poly I·C nor anti-IFN-α/β antiserum had a significant effect on the IgG1 response induced by MaMδ in the presence of anti-IFN-γ mAb. These observations demonstrate that the effects of poly I·C on Ig isotype selection in anti-δ plus anti-IFN-γ antibody treated mice, which closely resemble the effects of recombinant IFN-α, result from the stimulation of endogenous IFN-α and/or IFN-β production.

IFN-α/β Has a Relatively Modest Effect on Immunoglobulin (Ig) Isotype Secretion in Vitro by Lipopolysaccharide (LPS)-activated Splenic B Cells. To determine if IFN-α (or IFN-β, which binds to the same receptor) had any ability to influence Ig isotype selection in vitro in the absence of T lymphocytes, T cell-depleted spleen cells were cultured in vitro with LPS in the presence or absence of 10^4 U of recombinant mouse IL-4, ± 10 units of recombinant mouse IFN-γ or 1–10,000 U of recombinant mouse IFN-α. IgG1, IgG2a, and IgE levels in culture supernatants 6 d after the initiation of culture were

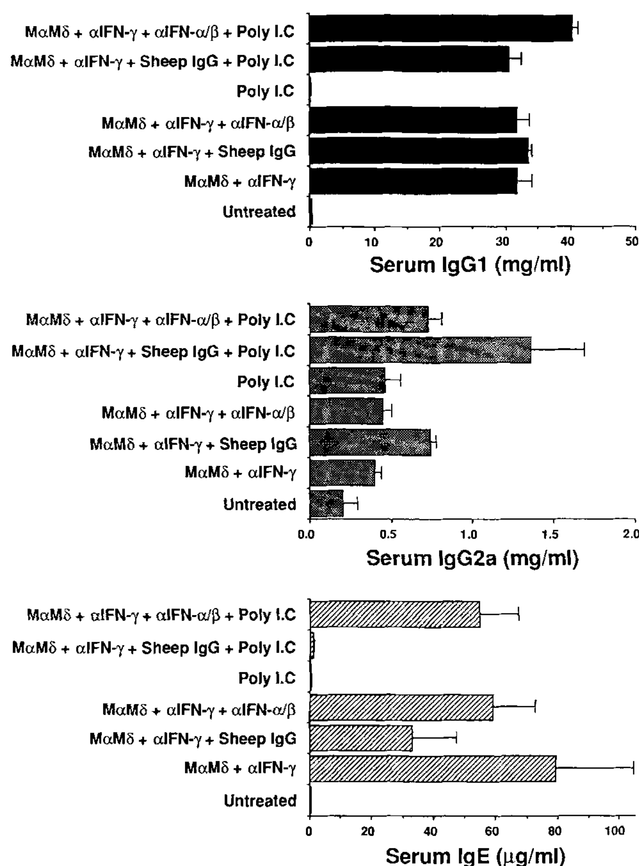

Figure 3. The inhibitory effect of poly I·C on the IgE response to MaMδ plus anti-IFN-γ mAb is blocked by anti-IFN-α/β antibody. BALB/c mice (5/group) were injected i.v. with 100 μg of each of two MaMδ mAbs (Hδ^a/1 and FF1-4D5) + anti-IFN-γ mAb, and were injected at the same time with 200 μg of poly I·C. Some groups of mice were injected i.v. 1 d before MaMδ injection with 0.1 ml of either normal sheep serum or sheep anti-mouse IFN-α/β antiserum. Additional groups of mice were left untreated or were injected with poly I·C in the absence of other treatment. Mice were bled 8 d after injection with MaMδ and/or poly I·C. Geometric means and standard errors of serum Ig levels are shown.

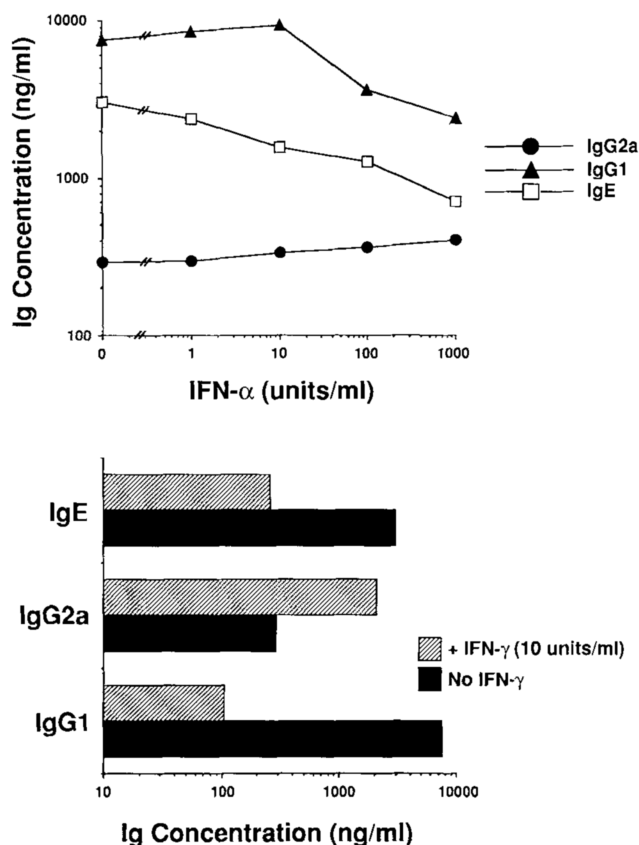

Figure 4. Effects of IFN-α and IFN-γ on Ig secretions by mouse B cells cultured with LPS ± IL-4. Small, T cell-depleted spleen cells were cultured with 20 μg/ml of LPS ± 10^4 U/ml of recombinant mouse IL-4 ± 10 U/ml of recombinant mouse IFN-γ (*lower panel*) or with the concentrations of recombinant mouse IFN-α shown (*upper panel*). After 6 d of culture supernatants were analyzed by ELISA for IgG1, IgG2a, and IgE content. IFN-α and IFN-γ inhibited IgG1 production by cells cultured with LPS in the absence of IL-4 to a similar extent as they inhibited IgG1 production by cells cultured with LPS + IL-4, although the addition of IL-4 increased IgG1 production over 50-fold. The addition of IL-4 to cell cultures reduced IgG2a production to barely detectable levels. No detectable IgE was produced in the absence of IL-4. Increasing the IFN-α concentration in cultures to 10^4 U/ml was highly toxic to cells, and suppressed all Ig production.

determined by ELISA (Fig. 4). At a concentration of 10 U/ml, IFN-α had no effect on the LPS-induced IgG2a response or the LPS + IL-4–induced IgG1 response, and inhibited the LPS + IL-4–induced IgE response by a factor of 2 (Fig. 4, upper panel). Increasing the concentration of IFN-α to 1,000 U/ml increased inhibition of the LPS + IL-4–induced IgG1 and IgE responses to factors of 3 and 4, respectively, but still had no significant effect on the LPS-induced IgG2a response. In contrast, IFN-γ at a concentration of 10 U/ml inhibited the LPS + IL-4–induced IgG1 and IgE responses by factors of 70 and 12, respectively, and enhanced the LPS-induced IgG2a response by a factor of 8 (Fig. 4, lower panel). Addition of anti-IFN-γ mAb to cultures inhibited the effects of IFN-γ, but not those of IFN-α (data not shown). Similar observations were made in additional in vitro experiments.

IFN-α Inhibits Splenic Interleukin (IL)-4 mRNA Levels and Enhances IFN-γ mRNA Levels in Anti-δ Antibody-treated Mice. The weak and similar inhibitory effects of IFN-α on IgG1 and IgE responses in an in vitro system, and the lack of a stimulatory effect of IFN-α on IgG2a production in this system, as compared to IFN-α's strong in vivo inhibition of IgE, but not IgG1 production, and stimulation of IgG2a production, suggested that IFN-α might indirectly inhibit in vivo IgE secretion by inhibiting the production of IL-4. This would be consistent with the observation that although IL-4 costimulates both IgG1 and IgE production and inhibits IgG2a production in vitro (8–11), anti-IL-4 and anti-IL-4 receptor mAbs completely block IgE production and enhance IgG2a production, but have little effect on IgG1 production in GaMδ-injected mice (12, 13). To investigate whether IFN-α might selectively modulate the production of IL-4 in vitro,

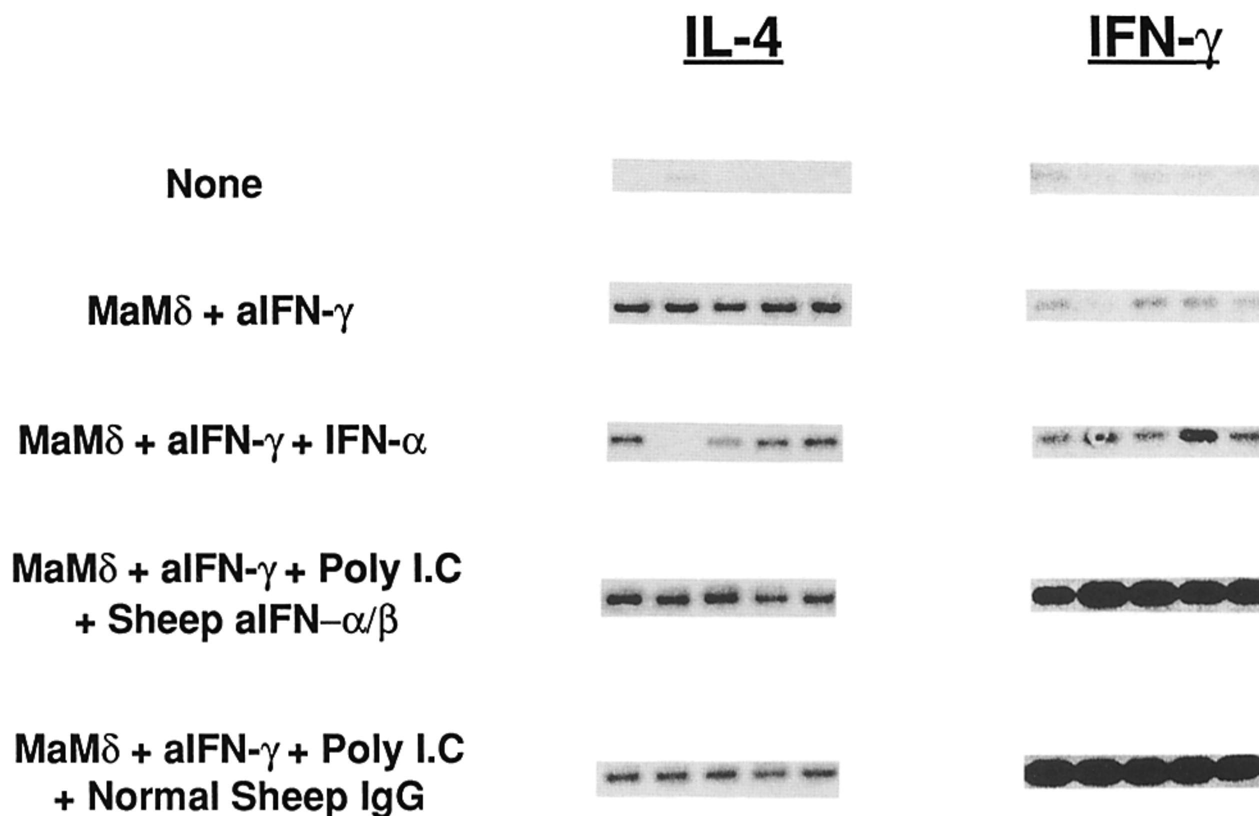

Figure 5. IFN-α inhibits the GaMδ-induced increase in splenic IL-4 mRNA levels and enhances the increase in splenic IFN-γ mRNA levels. BALB/c mice (5/group) were left untreated or were injected i.v. with 100 μg each of two anti-δ mAbs (Hδ[a]/1 and FF1-4D5), and 1 mg each of anti-FcγRII mAb (24G2) and anti-IFN-γ mAb (XMG-6). Some mice also were injected i.p. with 25,000 U of recombinant mouse IFN-α, twice daily, on the day of and 1 and 2 d after antibody administration. Two additional groups of mice that received the mAb cocktail were injected at the same time i.p. with 200 μg of poly I·C; one group was pretreated 1 d earlier with 0.2 ml of sheep anti–mouse IFN-α/β antiserum, the other group with a control sheep serum. mRNA was prepared from spleens 4 d after mAb injection. cDNA was prepared from mRNA and amplified with primers specific for IL-4 or IFN-γ DNA, as described in Materials and Methods, subjected to electrophoresis, and hybridized with specific probes by the Southern blot technique. The blots were exposed to phosphor screens and the resulting images were analyzed with a Molecular Dynamics phosphoimager.

we used a reverse transcriptase-PCR technique (32) to quantitate splenic IL-4 and IFN-γ mRNA levels in mice injected with MaMδ antibody plus anti–IFN-γ mAb in the presence or absence of recombinant mouse IFN-α. Levels were determined 4 d after anti-IgD antibody injection, because previous studies have shown that both cytokine genes are expressed at this time (32) and that both IL-4 and IFN-γ have an important effect at this time on Ig isotype selection (7, 39). Injection of mice with a combination of MaMδ, anti-FcγRII mAb, and anti–IFN-γ mAb induced 34- and 4-fold increases in the expression of splenic IL-4 and IFN-γ mRNA, respectively (Fig. 5), but had no effect on HPRT mRNA levels (data not shown). Injection of mice with 25,000 U of IFN-α twice daily on the day of and the 2 d after MaMδ injection decreased the level of splenic IL-4 mRNA by a factor of 4 and increased the splenic level of IFN-γ mRNA by a factor of approximately 3 (Fig. 5). Poly I·C, injected as a single 200 μg dose on the day of MaMδ injection also inhibited IL-4 gene expression; this effect was largely blocked if mice were pretreated with sheep anti–IFN-α/β antiserum rather than normal sheep IgG (Fig. 5). Poly I·C stimulated an approximately nine-fold increase in IFN-γ gene expression, regardless of whether mice were pretreated with anti–IFN-α/β or control antiserum. This is consistent with our previous observation that poly I·C directly stimulates IFN-γ, as well as IFN-α secretion (Snapper, C.M., H. Yamaguchi, J.F. Urban, Jr., and F.D. Finkelman, manuscript submitted for publication).

Discussion

The discoveries that two cytokines, IL-4 and IFN-γ, can have striking, and usually opposite effects on Ig isotype selection (1, 2, 11), and that normal $CD4^+$ T cells and T cell clones can differ markedly in their relative secretion of these two cytokines (3), raised two additional questions: (*a*) Are

there additional cytokines that regulate Ig isotype selection? and (*b*) What regulates the differentiation of virgin, resting $CD4^+$ T cells into effector cells that secrete high or low ratios of IL-4/IFN-γ. The data presented in this paper make it likely that IFN-α (and presumably, IFN-β), is in the group of cytokines that regulates Ig isotype selection indirectly by decreasing the ratio of IL-4/IFN-γ that is secreted by T cells as well as through a direct effect on B cells. This interpretation is supported by observations that: (*a*) recombinant IFN-α inhibits IgE secretion and enhances IgG2a secretion when injected into anti-IgD antibody-injected mice; (*b*) these effects are not IFN-γ dependent, since they are observed in mice injected with an anti–IFN-γ mAb; (*c*) the effect of IFN-α is mimicked by poly I·C, which stimulates endogenous IFN-α secretion; (*d*) a sheep antiserum, that neutralizes IFN-α and IFN-β, blocks the Ig isotype modifying effects of poly I·C in mice stimulated with a combination of anti-IgD and anti–IFN-γ mAbs; (*e*) IFN-α is considerably less potent than IFN-γ in its inhibition of IgG1 and IgE production by LPS + IL-4–stimulated B cells in vitro, and, unlike IFN-γ, has no ability to enhance IgG2a production by LPS-activated B cells; and (*f*) IFN-α treatment of anti-IgD antibody-treated mice decreases the splenic level of IL-4 mRNA but increases the level of splenic IFN-γ mRNA. The observation that IFN-α inhibits in vivo IgG1 production in the absence of anti–IFN-γ mAb, but has no effect on IgG1 production in the presence of anti–IFN-γ mAb, suggests that the in vivo effect of IFN-α on this isotype is entirely due to the stimulation of increased IFN-γ production.

Our overall interpretation must be made, however, with some caveats: (*a*) Anti-IgD antibody is an atypical immunogen. It binds to, and is processed and presented to T cells by, the majority of B cells, with the result that T cell activation and cytokine secretion are greatly enhanced as compared to the levels that are usually observed during a response to a conventional T cell-dependent antigen. This large degree of T cell activation contributes to the generation of an enormous Ig response in anti-IgD antibody-treated mice (30, 31). While these peculiarities of the anti-IgD antibody system make it ideal for the detection of cytokine effects on Ig isotype and cytokine gene expression, there is no assurance that IFN-α will have the same effects in responses to conventional antigens that it has in anti-IgD antibody-injected mice. It is reassuring, however, that the effects of IFN-γ and IL-4 on Ig isotype selection that were initially observed in GaMδ antibody-injected mice have since been confirmed in other systems (2). (*b*) Since no mAbs that potently neutralize both IFN-α and IFN-β are available, it was necessary to use a polyclonal sheep anti–IFN-α/β antibody in studies performed to demonstrate that the effect of poly I·C on Ig isotype selection resulted from IFN-α/β production. Since this sheep antibody was prepared against purified natural mouse IFN-α and IFN-β, it is impossible to be completely certain that it does not have some ability to neutralize cytokines in addition to IFN-α/β. Tests have demonstrated, however that it lacks anti–IL-2, IFN-γ, or TNF antibody activity. (*c*) Our observation that IFN-α treatment inhibits splenic IL-4 mRNA levels, and enhances IFN-γ mRNA levels, demonstrates that IFN-α differentially regulates cytokine gene expression. It does not, however, prove that IFN-α modifies the secretion of these cytokines to the same extent, since protein synthesis and secretion may be at least partially controlled at translational or posttranslational levels. (*d*) In as much as we have quantitated mRNA levels in unfractionated spleen cells, we cannot identify with certainty the cell population or populations in which IFN-α affected IL-4 and IFN-γ gene expression. It seems likely, however, that the effects observed were predominantly in $CD4^+$ T cells, since T cells are the only cells in GaMδ-treated mice that secrete detectable IL-4 (40) and since $CD4^+$ spleen cells from these mice contain greater than 100-fold more IL-4 encoding mRNA than do $CD4^-$ spleen cells (32).

Our interpretation draws support from the observation that most viruses, which induce the production of large quantities of IFN-α/β as well as IFN-γ, induce the production of IgG2a as their predominant Ig isotype (41). Studies are currently being performed to determine the relative contributions of IFN-α/β and IFN-γ to IgG2a predominance in these mice. A role for IFN-α/β in the determination of T cell differentiative pathways is also compatible with results of investigations of mice infected with the protozoan parasite *Leishmania major*. T cells of BALB/c mice infected with this parasite secrete a high ratio of IL-4/IFN-γ, while T cells of *Leishmania*-infected C57BL/6 mice secrete a high ratio of IL-4/IFN-γ ratio (6). This difference in cytokine secretion is at least partially responsible for the progressive disease observed in BALB/c mice, as opposed to the limited disease seen in C57BL/6 mice, as well as for the expected differences in the Ig isotypes secreted by these different mouse strains (5, 6). Interestingly, macrophages from BALB/c mice produce much less IFN-α than do C57BL/6 macrophages, when stimulated with agents such as poly I·C (42). Experiments are being performed to determine whether this correlation is fortuitous or reflective of an important control mechanism in the establishment of different T cell cytokine secretory patterns.

IFN-α is not the only factor that can influence the ratio of IL-4/IFN-γ secretion by T lymphocytes. IFN-γ inhibits the proliferation of T cell clones that secrete IL-4, IL-5, and IL-10 (Th2 cells) but not the proliferation of T cell clones that secrete IL-2 and IFN-γ (Th1 cells) (15). IL-10 inhibits cytokine production by Th1, but not by Th2 cells (16). Neither IFN-γ nor IL-10, however, has yet been shown to influence the differentiation of uncommitted, resting T cells in vitro or to influence T cell cytokine production in vivo. In contrast, while IL-4 has not been shown to affect cytokine secretion by established Th1 or Th2 cells, it promotes the differentiation of T cells into IL-4–secreting cells in some in vitro systems, and appears to directly or indirectly promote IL-4 secretion and inhibit IFN-γ secretion in some in vitro and in vivo systems (43–46).

IFN-α differs from IL-4, IL-10, and IFN-γ in that it is produced predominantly by non-T cells, and especially by a cell type, the macrophage (37, 38), that has an important role in the presentation of antigen to T cells. Thus, the capacity of an antigen to stimulate macrophage production of IFN-α,

which can occur soon after macrophage and antigen first come into contact, may allow virgin T cells to simultaneously be presented with processed antigen, that leads to their activation, plus a cytokine that induces them to differentiate in a Th1 direction. However, while IL-4, IL-10, and IFN-γ appear to be produced predominantly by T cells, their production by other cell types has also been described. We would propose that B cell production of IL-10 (47), NK cell production of IFN-γ (48), mast cell or basophil production of IL-4 (49–51), and the presumed production of other cytokines by non-T cells early in the course of an immune response may, like macrophage production of IFN-α, act to influence the direction of T cell differentiation. This hypothesis would suggest that auxiliary properties of antigens, that lead to their stimulation of specific cytokines by non-T cells, rather than the expression of specific T cell epitopes, regulates differential T cell cytokine production.

We thank Dr. Jacques Banchereau for his many valuable suggestions.

This work was supported in part by National Institutes of Health grants AI-21328 and AI-26150, Naval Medical Research and Development Command grants N0007590WR0008 and N0007590WR00097, and Uniformed Services University of the Health Sciences Research Protocols R083AB and R074CN. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

Address correspondence to Fred D. Finkelman, Department of Medicine, Room A3060, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.

Received for publication 15 April 1991 and in revised form 8 July 1991.

References

1. Coffman, R.L., B.W. Seymour, D.A. Lebman, D.D. Hiraki, J.A. Christiansen, B. Shrader, H.M. Cherwinski, H.F.J. Savelkoul, F.D. Finkelman, M.W. Bond, and T.R. Mosmann. 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* 102:5.
2. Finkelman, F.D., J. Holmes, I.M. Katona, J.F. Urban, Jr., J.P. Beckmann, L.S. Park, K.A. Schooley, R.L. Coffman, T.R. Mosmann, and W.E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8:303.
3. Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: Different patterns of lymphokines secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145.
4. Bottomly, K. 1988. A functional dichotomy in CD4 T lymphocytes. *Immunol. Today.* 9:274.
5. Scott, P., P. Natovitz, R. Coffman, E. Pearce, and A. Sher. 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. Exp. Med.* 168:1675.
6. Heinzel, F.P., M.D. Sadick, B.J. Holaday, R.L. Coffman, and R.M. Locksley. 1989. Reciprocal expression of interferon γ or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* 169:59.
7. Finkelman, F.D., I.M. Katona, T.R. Mosmann, and R.L. Coffman. 1988. Interferon-γ regulates the isotypes of immunoglobulin secreted during in vivo humoral immune responses. *J. Immunol.* 140:1022.
8. Vitetta, E.S., J. Ohara, C. Myers, J. Layton, P.H. Krammer, and W.E. Paul. 1985. Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. *J. Exp. Med.* 161:1726.
9. Coffman, R.L., and J. Carty. 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J. Immunol.* 136:949.
10. Coffman, R.L., J. Ohara, M.W. Bond, J. Carty, A. Zlotnik, and W.E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4538.
11. 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.
12. Finkelman, F.D., I.M. Katona, J.F. Urban, Jr., J. Holmes, J. Ohara, A.S. Tung, J. vG. Sample, and W.E. Paul. 1988. Interleukin-4 is required to generate and sustain in vivo IgE responses. *J. Immunol.* 141:2335.
13. Finkelman, F.D., J.F. Urban, Jr., M.P. Beckmann, K.A. Schooley, J.M. Holmes, and I.M. Katona. 1991. Regulation of murine in vivo IgG and IgE responses by a monoclonal anti-IL-4 receptor antibody. *Internatl. Immunol.* 3:599.
14. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone: 1. Definition according to the profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
15. Gajewski, T.F., and F.W. Fitch. 1988. Anti-proliferative effect of IFN-γ in immune regulation. I. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T cell clones. *J. Immunol.* 140:4245.
16. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann. 1989. Two types of mouse helper T cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170:2081.
17. Smith, T.J., and R.R. Wagner. 1967. Rabbit macrophage in-

terferons. I. Conditions for biosynthesis by virus-infected and uninfected cells. *J. Exp. Med.* 125:559.
18. Pene, J., F. Rousset, F. Briere, I. Chretien, J.Y. Bonnefoy, H. Spits, T. Yokota, K. Arai, J. Banchereau, and J. de Vries. 1988. IgE production by normal human lymphocytes is induced by interleukin-4 and suppressed by interferons γ and α and prostaglandin E2. *Proc. Natl. Acad. Sci. USA.* 85:6880.
19. Youngner, J.S., and W.R. Stinebring. 1964. Interferon production in chickens infected with Brucella abortus. *Science (Wash. DC).* 144:1022.
20. Desoner, P., E. DeClercq, C. Cocito, and A. Billiau. 1970. The interferon inducer from Brucella. *Ann. N.Y. Acad. Sci.* 170:247.
21. Finkelman, F.D., S.W. Kessler, J.F. Mushinski, and M. Potter. 1981. IgD secreting murine plasmacytomas: identification and characterization of two IgD myeloma proteins. *J. Immunol.* 126:680.
22. Goroff, D.K., A. Stall, J.J. Mond, and F.D. Finkelman. 1986. In vitro and in vivo B lymphocyte activating properties of monoclonal anti-δ antibodies. I. Determinants of B lymphocyte activating properties. *J. Immunol.* 135:2381.
23. Zitron, I.M., and B.L. Clevinger. 1980. Regulation of murine B cells through surface immunoglobulin. I. Monoclonal anti-δ antibody that induces allotype-specific proliferation. *J. Exp. Med.* 152:1135.
24. Cherwinski, H.C., J.H. Schumacher, K.D. Brown, and T.R. Mosmann. 1987. Two types of mouse helper T cell clone. 3. Further differences in lymphokine synthesis between TH1 and TH2 clones revealed by RNA hybridization, functionally monospecific bioassays and monoclonal antibodies. *J. Exp. Med.* 166:1229.
25. Unkeless, J.C. 1979. Characterization of a monoclonal antibody against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* 153:1198.
26. Gresser, I., M.G. Tovey, M.T. Bandu, C. Maury, and D. Brouty-Boye. 1976. Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. I. Rapid evolution of encephalomyocarditis virus infection. *J. Exp. Med.* 144:1305.
27. Ohara, J., and W.E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature (Lond.).* 315:333.
28. Hu-Li, J., J. Ohara, C. Watson, W. Tsang, and W.E. Paul. 1989. Derivation of a T cell line that is highly responsive to IL-4 and IL-2 (CT.4R) and of an IL-2 hyporesponsive mutant of that line (CT.4S). *J. Immunol.* 142:800.
29. Ledbetter, J.A., and L.A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.
30. 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.
31. Finkelman, F.D., C.M. Snapper, J.D. Mountz, and I.M. Katona. 1987. Polyclonal activation of the murine immune system by a goat antibody to mouse IgD. IX. Induction of a polyclonal IgE response. *J. Immunol.* 139:2172.
32. Svetić, A., F.D. Finkelman, C.W. Dieffenbach, D.E. Scott, A.D. Steinberg, and W.C. Gause. 1991. Cytokine gene expression following in vivo primary immunization with GaMδ antibody. *J. Immunol.* In press.
33. Cerottini, J.-C., and H.R. MacDonald. 1989. The cellular basis of T-cell memory. *Annu. Rev. Immunol.* 7:77.
34. Diamond, S.L., L.V. McIntire, J.B. Sharefkin, C. Dieffenbach, K. Frasier-Scott, and S.G. Eskin. 1990. Tissue plasminogen activator mRNA levels increase in cultured human endothelial cells exposed to laminar sheer stress. *J. Cell. Physiol.* 143:364.
35. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 545.
36. Field, A.K., A.A. Tytell, G.P. Lampson, and M.R. Hilleman. 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc. Natl. Acad. Sci. USA.* 58:1004.
37. Maehara, N., and M. Ho. 1977. Cellular origin of interferon induced by bacterial lipopolysaccharide. *Infect. Immun.* 15:78.
38. Havell, E.A., and G.L. Spitalny. 1983. Endotoxin-induced interferon synthesis in macrophage cultures. *J. Reticuloendothel. Soc.* 33:369.
39. Finkelman, F.D., J. Holmes, J.F. Urban, Jr., W.E. Paul, and I.M. Katona. 1989. T help requirements for the generation of an in vivo IgE response: A late acting form of T cell help other than IL-4 is required for IgE but not for IgG1 production. *J. Immunol.* 142:403.
40. Finkelman, F.D., J. Ohara, D.K. Goroff, J. Smith, N. Villacreses, J.J. Mond, and W.E. Paul. 1986. Production of BSF-1 during an in vivo, T-dependent immune response. *J. Immunol.* 137:2878.
41. Coutelier, J.P., J.T.M. van der Logt, F.A. Heessen, G. Warnier, and J. Van Snick. 1987. IgG2a restriction of murine antibodies elicited by viral infections. *J. Exp. Med.* 165:64.
42. De Maeyer, E., M.-C. Hoyez, J. De Maeyer-Guignard, and D.W. Bailey. 1979. Effect of mouse genotype on interferon production. III. Expression of IF-1 by peritoneal macrophages in vitro. *Immunogenetics.* 8:257.
43. Le Gros, G., S.Z. Ben-Sasson, R. Seder, F.D. Finkelman, and W.E. Paul. 1990. Generation of interleukin 4 (IL-4)–producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4–producing cells. *J. Exp. Med.* 172:921.
44. Swain, S.L., A.D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145:3796.
45. Wagner, F., N. Fischer, C. Lersch, R. Hart, and H. Dancygier. 1989. Interleukin 4 inhibits the interleukin 2-induced production of its functional antagonist, interferon gamma. *Immunol. Letters.* 21:237.
46. Sadick, M.D., F.P. Heinzel, B.J. Holaday, R.T. Pu, R.S. Dawkins, and R.M. Locksley. 1990. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon γ-independent mechanism. *J. Exp. Med.* 170:115.
47. O'Garra, A., G. Stapleton, V. Dhar, M. Pearce, J. Schumacher, H. Rugo, D. Barbis, A. Stall, J. Cupp, K. Moore, P. Vieira, T. Mosmann, A. Whitmore, L. Arnold, G. Haughton, and M. Howard. 1990. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Internatl. Immunol.* 2:821.
48. Brunswick, M., and P. Lake. 1985. Obligatory role of gamma-interferon in T cell-replacing factor-dependent, antigen-specific murine B cell responses. *J. Exp. Med.* 161:953.
49. Brown, M.A., J.A. Pierce, C.J. Watson, J. Falco, J.N. Ihle, and W.E. Paul. 1987. B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. *Cell.* 50:809.
50. Plaut, M., J.H. Pierce, C.J. Watson, J. Hanley-Hyde, R.P.

Nordan, and W.E. Paul. 1989. Mast cell lines produce lymphokines in responses to cross linkage of FcεRI or to calcium ionophores. *Nature (Lond.).* 339:64.

51. Seder, R.A., W.E. Paul, A.M. Dvorak, S.J. Sharkis, A. Kagey-Sobotka, Y. Niv, F.D. Finkelman, S.A. Barbieri, S.J. Galli, and M. Plaut. 1991. Mouse splenic and bone marrow cell populations that express high affinity Fcε receptors and produce IL-4 are highly enriched in basophils. *Proc. Natl. Acad. Sci. USA.* 88:2835.