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The Prototypic Th2 Autoimmunity Induced by Mercury Is Dependent on IFN-γ and Not Th1/Th2 Imbalance[1]

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Imbalances of Th1- and Th2-type responses have been postulated to be a predisposing factor for both humoral and cellular mediated autoimmune diseases. To further define their roles in systemic autoimmunity, IL-4 and IFN-γ gene knockout mice were studied for susceptibility to the prototypic Th2-mediated mercury-induced autoimmunity. A predominant Th2-type response following $HgCl_2$ treatment of wild-type B10.S mice was confirmed by the findings of a significant increase in splenic IL-4 and hypergammaglobulinemia primarily of the IgG1 isotype, without an increase in IFN-γ levels. Paradoxically, IL-4-deficient mice developed the characteristic anti-nucleolar autoantibodies and tissue deposition of immune complexes, while IFN-γ-deficient mice had very low autoantibody levels and essentially normal immunohistology. Studies to define defects in Ab responses of IFN-γ-deficient mice, using the T-dependent Ag (4-hydroxy-3-nitrophenyl)acetyl, revealed an attenuated IgG response to low and to a lesser extent high doses of (4-hydroxy-3-nitrophenyl)acetyl-hemocyanin, but maintenance of affinity maturation. These results indicate that Th1/Th2 imbalance does not directly play a role in susceptibility to mercury-induced autoimmunity, and suggest that the dependence on Th1-type responses in certain autoimmune diseases is due to the requirement for IFN-γ for Ab production to weakly antigenic self molecules. ***The Journal of Immunology,*** **1998, 161: 234–240.**

Following activation, $CD4^+$ T cells become polarized into two main Th cell types, designated Th1 and Th2 (1, 2). Th1 cells produce IFN-γ, IL-2, and TNF-β; express surface IL-12Rβ (3, 4); and are considered to promote cellular immune responses. In contrast, Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13; express the IL-4-related GATA-3 transcription factor (5), IFN-γRβ (6), and the CCR3 chemokine receptor (7); and are thought to promote humoral immune responses. Because of their functional dichotomy, dominance of one or the other Th type in an immune reaction has been postulated to result in either a predominantly cellular (Th1-mediated) or predominantly Ab (Th2-mediated) response. Although in most instances immune responses most likely involve both Th types, shifts toward mainly Th1- or Th2-type responses have been documented in vitro (1) and in vivo (8, 9), and a number of factors that promote either Th1 or Th2 responses have been identified (10, 11). Such skewing of the type of Th response has been shown to play important roles in resistance to certain infections, in transplantation rejection, and in susceptibility to autoimmunity (8, 12–14).

One of the autoimmune models in which Th1/Th2 imbalance has long been considered to play a critical role is $HgCl_2$-induced autoimmunity (HgIA)[3] (15, 16). Clinical features of this disease, which follow nonlethal exposure to various forms of mercury, are similar to spontaneous systemic lupus erythematosus and include lymphoproliferation, hypergammaglobulinemia, autoantibody production, and immune complex-mediated tissue injury (reviewed in Ref. 17). Other similarities are the requirements for T cells (18) and for CD40/CD40 ligand and B7/CD28 costimulatory molecule interactions to generate autoantibodies (19). HgIA susceptibility is also strikingly influenced by the MHC with particular class II haplotypes determining autoantibody specificity and disease severity (20).

Considerable evidence suggests that HgIA is mediated by the Th2 subset. Murine HgIA is associated with increases of predominantly IL-4 and IL-10, with only small early increases in IFN-γ and IL-2 (15, 19, 21–23). $HgCl_2$-induced polyclonal activation of B cells results in hypergammaglobulinemia, primarily of the Th2-related IgG1 and IgE isotypes (15). Studies based on IL-4 and IFN-γ cytokine profiles have suggested that Th2-type responses promote HgIA, while Th1-type responses are inhibitory (22, 24, 25). Treatment of susceptible rats with Ab to the Th1-type subset ($OX22^+$) before administration of $HgCl_2$ leads to exacerbation of the autoimmune syndrome (26). Simultaneous administration of $HgCl_2$ protects against the induction of Th1-mediated autoimmune diseases, such as experimental autoimmune uveoretinitis (27) and experimental allergic encephalomyelitis, presumably by promoting Th2-type responses (28). Finally, in the susceptible BN rat, induction of HgIA can be suppressed by anti-self MHC class II T cells that are of the Th1 type (29). Despite these findings, other studies using more direct approaches have been unable to demonstrate a critical role for Th2 cells. Treatment of $HgCl_2$-treated animals with anti-IL-4 mAb suppressed total IgE levels and IgG1 isotype anti-nucleolar Abs (ANoA), but not total IgG ANoA titers (21). rIFN-γ therapy was not protective against ANoA production

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[3] Abbreviations used in this paper: HgIA, mercury-induced autoimmunity; ANoA, anti-nucleolar Ab; HPRT, hypoxanthine-guanine phosphoribosyltransferase; NP, (4-hydroxy-3-nitrophenyl)acetyl.

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or glomerular immune complexes (30), and anti-IFN-γ treatment only transiently and marginally augmented proteinuria in young mice (25). Although there may be technical explanations for the lack of response to these interventions, these findings nonetheless question the current paradigm that Th2 promotes and Th1 suppresses HgIA.

In contrast to the findings in HgIA, recent studies have indicated that spontaneous systemic autoimmunity is highly dependent on Th1 cytokines. In vivo cytokine profiles in the lupus-prone MRL-*lpr* and BXSB strains showed increase in the Th1 cytokine, IFN-γ (31). Greater increases in the IgG2a and IgG3 (Th1-mediated) than IgG1 isotypes (Th2-mediated) were observed in susceptible MRL-*lpr* and MRL.Yaa compared with less susceptible MRL-+/+ and MRL-*lpr*-ll (long-lived substrain of MRL-*lpr*) strains (32). Treatment with IFN-γ accelerates disease, whereas anti-IFN-γ or soluble IFN-γR treatment prevented systemic autoimmune disease (33, 34), and IL-4 transgenic lupus-prone (NZW × C57BL/6.Yaa)F_1 mice have attenuated disease (35). MRL-*lpr* mice deficient of IFN-γ (36) or IFN-γR (37) by homologous recombinant gene knockout had lower IgG2a and IgG3 autoantibodies associated with less end organ disease. These studies clearly support a primary role for Th1-type responses in systemic autoimmunity. However, the situation may be more complex since MRL-*lpr* mice homozygous for the IL-4 gene knockout had levels of IgG2a and IgG3 isotypes and autoantibodies comparable with wild-type MRL-*lpr* mice, but had reduced glomerulonephritis (36).

To more directly study the roles of the Th2 and Th1 subsets in HgIA, mice with deletions of IL-4 or IFN-γ genes were examined for disease susceptibility. In contrast to what would be predicted, IL-4 gene knockout mice were as susceptible to HgIA as wild-type mice, whereas IFN-γ-deficient mice were resistant to HgIA, at the level of autoantibody production. These findings suggest that IFN-γ, rather than an imbalance of Th1 and Th2, is important for susceptibility to autoimmunity.

Materials and Methods

Animals

Breeding and maintenance of mice with targeted disruption of the IFN-γ gene (IFN-$\gamma^{-/-}$, Ref. 38) or the IL-4 gene (IL-$4^{-/-}$, Ref. 39) were performed under specific-pathogen-free conditions at The Scripps Research Institute Animal Facility (La Jolla, CA). H-2^s haplotype knockout mice were generated by first backcrossing homozygous knockout mice (H-2^b haplotype) for two generations to B10.SeCD1 (H-2^s) mice. The (IFN-$\gamma^{-/-}$ × B10.S)F_1 × B10.S and (IL-$4^{-/-}$ × B10.S)F_1 × B10.S N2 offspring were then typed for H-2^s and the appropriate disrupted gene. Mice, both homozygous for H-2^s and heterozygous for the recombinant knockout gene (~1/4 of mice), were then intercrossed to generate the +/+, +/−, and −/− mice. H-2 typing was performed by PCR of genomic DNA using the D17 Mit16 primers (Research Genetics, Huntsville, AL) that are polymorphic for H-2^b and H-2^s haplotypes. IFN-γ and IL-4 wild-type and knockout genes were typed by PCR of genomic DNA (35 cycles of 94°C/20 s, 60°C/30 s, 72°C/90 s). Two pairs of primers were used for typing IFN-γ, one detecting the undisrupted second exon (sense, AGAAGTAAGTG GAAGGGCCCAGAAG; antisense, AGGGAAACTGGGAGAGGAGAA ATAT; ~200 bp) and the other the neomycin gene (sense, TTGAACAA GATGGATTGCACGCAGG; antisense, GCCTGGCGCGAGCCCCTGA TGCTCT; ~400 bp). A single pair of primers was used for typing IL-4 (sense, CTGCCAGCATTGCATTGTTA; antisense, ATGGTGCCAGAT AGGTACTT; wild-type ~200 bp, and knockout ~1 kb).

Mercury treatment

Six- to eight-week-old mice were injected twice per week for 4 wk, or as indicated, s.c. with 40 μg $HgCl_2$ in 100 μl PBS, as previously described (40). Controls received PBS alone.

Serology

Anti-nuclear Ab. Indirect immunofluorescence was performed on sera, as previously described (40). Briefly, monolayers of HEp-2 cells on slides (Bion Enterprises, Park Ridge, IL) were incubated with serial twofold dilutions of mouse serum starting from 1/100, followed by a 1/100 dilution of FITC-conjugated goat anti-mouse IgG + IgM Abs (Caltag Laboratories, Burlingame, CA). Anti-nuclear Ab patterns and titers were assessed under blinded conditions. Anti-chromatin Abs were measured by ELISA, as previously described (41, 42).

Ig levels. Serum IgG and IgG1 levels were quantitated, as previously described (43). ELISA plates were coated with 200 μl of 2 μg/ml goat anti-mouse κ Ab (Caltag) diluted in PBS and incubated overnight at 4°C. Plates were postcoated for 1 h with 0.1% gelatin in PBS, followed by washing with PBS/0.05% Tween-20. A total of 200 μl of diluted sera was incubated in duplicates while shaking for 2.5 h, followed by washing three times with PBS/0.05% Tween-20. A standard curve was generated by serial dilutions of 400 to 3.125 ng/ml of polyclonal mouse reference sera (The Binding Site, Birmingham, U.K.). Horseradish peroxidase-conjugated goat anti-mouse IgG or IgG1 Abs (Caltag) were diluted 1/5000 or 1/6000, respectively, in anti-Ig diluent and incubated while shaking for 90 min. Following washing three times with PBS/0.05% Tween and washing four times with PBS, substrate solution was added and OD 405 nm was measured. Serum IgG2a levels were similarly quantitated with the following modifications. Plates were coated with rat anti-mouse Ig κ (PharMingen, San Diego, CA), and reagents that detected both the a and b allotypes of IgG2a were used throughout the assay, since some mice had the IgH^a allotype from 129 strain (ES cell derived). The horseradish peroxidase-conjugated rat anti-mouse IgG2a (PharMingen) Ab was diluted 1/1000. Standard curves were generated by serial dilutions of purified mouse IgG2a of both allotypes (PharMingen). IgG, IgG1, and IgG2a concentrations were extrapolated from their standard curves.

Anti-(4-hydroxy-3 nitrophenyl)acetyl (NP) Ab response

Mice were immunized i.p. with 100 μl of PBS containing varying amounts of ALUM-precipitated NP-conjugated hemocyanin (*Limulus* polyphemus; Sigma, St. Louis, MO) (20:1 ratio). ELISA using BSA and NP-conjugated BSA at ratios of 20:1 and 2.5:1 were used as substrates to assess NP-specific Ab levels and affinity maturation. Briefly, 100 μl of Ag at 2 μg/ml in PBS was incubated overnight at 4°C. After washing, wells were blocked with 200 μl of gelatin for 1.5 h, washed with PBS/Tween-20, then incubated with serum diluted 1/300 in diluent buffer for 1.5 h. After washing, 100 μl of horseradish peroxidase-conjugated Ab (goat anti-mouse IgM at 1/3,000 dilution or goat anti-Igλ chain at 1/25,000 dilution; Southern Biotechnology, Birmingham, AL) was then added, and after 1 h, wells were washed and incubated with substrate, as described above for IgG quantitation. Positive and negative control sera were used to standardize assays, and the NP-specific responses were obtained after subtracting for responses to BSA (<0.005 OD over background).

Immunoprecipitation

Immunoprecipitation was performed, as previously described (40), using in vitro translated mouse fibrillarin (44). Briefly, 100 μl of protein A-Sepharose CL4B beads were incubated with 5 μl of serum in 500 μl of NET2 + F buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) for 1 h at 4°C, then washed and incubated for 1.5 h with 10 μl of radiolabeled translation product in 1 ml of NET2 + F buffer. Beads were then washed, resuspended in 2× sample loading buffer, and boiled. Supernatants were electrophoresed on polyacrylamide gels, and precipitates were identified by autoradiography.

Immunohistology

Sections of kidney and spleen were stained for direct immunofluorescence, as previously described (45). Briefly, 4-μm cryostat sections were ethanol fixed and incubated with serial dilutions of FITC-conjugated goat anti-mouse IgG Abs (Southern Biotechnology). The endpoint titer of the glomerular deposits was defined as the highest dilution of Ab at which a specific fluorescence could be detected. Positive immunofluorescence with titers less than 1:40 were considered background. Vessel wall IgG deposits were graded on a 0 to 4^+ scale. Slides were examined under blinded conditions.

Cytokine competitive RT-PCR

The procedure to determine IL-4, IFN-γ, and hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA levels by a competitive RT-PCR assay has been previously described (31). This approach quantitates the amount of cDNA through the use of an internal competitor standard of slightly different size than the cDNA. Quantitation is obtained by coamplifying cDNA with known amounts of the competitor standard in single reaction tubes and then comparing the PCR signals of the cDNA and the

Table I. *Total serum IgG, IgG1, and IgG2a levels in $HgCl_2$-treated IL-4-deficient mice*[a]

Mice	No.	Treatment	Total IgG		IgG1		IgG2a	
			Prebleed	Postbleed	Prebleed	Postbleed	Prebleed	Postbleed
IL-4^{++}	7	PBS	625 ± 240	1010 ± 311	78 ± 22	180 ± 37	36 ± 20	88 ± 34
IL-4^{++}	12	$HgCl_2$	1758 ± 871	3714 ± 432[c]	332 ± 223	1233 ± 218[b]	81 ± 66	157 ± 34
IL-$4^{+/-}$	6	PBS	1888 ± 471	1981 ± 327	180 ± 44	237 ± 51	97 ± 23	238 ± 35
IL-$4^{+/-}$	9	$HgCl_2$	1169 ± 144	4964 ± 820[b]	141 ± 50	1561 ± 437[b]	44 ± 11	261 ± 53
IL-$4^{-/-}$	2	PBS	944 ± 53	1633 ± 48	6 ± 1	3 ± 0.2	236 ± 45	421 ± 29
IL-$4^{-/-}$	8	$HgCl_2$	1793 ± 574	3712 ± 1083	18 ± 6	52 ± 8[b]	136 ± 40	410 ± 102[d]

[a] Ig levels are expressed as mean ± SE in micrograms per milliliter for the number of mice indicated. Pre- and postbleed sera were obtained at 0 and 4 wk of treatment, respectively. Serum Igs were quantitated by ELISA (see *Materials and Methods*).
[b] $p = 0.003$, [c] $p < 0.0001$ between $HgCL_2$ and PBS treatment groups.
[d] $p = 0.013$ for IL-$4^{+/+}$ vs IL-$4^{-/-}$ groups treated with $HgCl_2$.

internal standard. Briefly, 2 μg of total cellular splenic RNA was reverse transcribed to generate cDNA. Aliquots containing a set amount of cDNA, specific primers, and serial dilutions of the competitor plasmid were subjected to PCR. The primer sequences, PCR conditions, and the competitor plasmid containing internal standards for 14 cytokine or control sequences, including IL-4, IFN-γ, and HPRT, have been described in detail (31). Products were run on 2% agarose gels, stained with ethidium bromide, and quantitated using a digital video imaging system (Eagle Eye II; Stratagene, La Jolla, CA). Quantity of mRNA was calculated by linear regression analysis and expressed relative to HPRT levels using the formula: 1000 × [(quantity of IL-4 or IFN-γ)/(quantity of HPRT)].

Statistics

Data were analyzed by the unpaired two-tailed *t* test or Mann-Whitney *U* test (StatView; Abacus Concepts, Berkeley, CA). Differences were considered statistically significant at $p < 0.05$.

Results

IL-4 is increased in $HgCl_2$-induced autoimmunity

In vivo IL-4 and IFN-γ production in spleens of autoimmune-prone B10.S mice treated with the typical regimen of $HgCl_2$ for 4 wk was compared with those of PBS-injected controls using a competitive RT-PCR assay (31). Splenic IL-4 expression in $HgCl_2$-treated mice was nearly four times higher than in PBS controls (10.9 ± 1.9 vs 2.8 ± 0.9, $p = 0.003$; 8 mice in each group), whereas mean IFN-γ levels, although higher in $HgCl_2$-treated animals, did not reach statistical significance because of considerable individual variability (40.8 ± 36.1 vs 19.2 ± 5, $p > 0.05$; 8 mice in each group). These findings along with previous reports (19, 46) support the contention that IL-4 and, therefore, Th2 cells play an important role in the generation of autoimmunity in this model.

$HgCl_2$ treatment of IL-$4^{-/-}$ mice

To directly examine the role of IL-4 and Th2 cells in this disease, H-2^s mice deficient in IL-4 by homologous recombinant gene knockout (IL-$4^{-/-}$) (39) were examined for HgIA. Three types of IL-4 mice, including wild-type (IL-$4^{+/+}$), heterozygous knockout (IL-$4^{+/-}$), and homozygous knockout (IL-$4^{-/-}$), were treated for 4 wk with either the standard $HgCl_2$ regimen or PBS.

As shown in Table I, prebleed total IgG levels were similar in all three IL-4 groups, whereas very low IgG1 and slightly higher IgG2a levels were observed in the IL-$4^{-/-}$ mice compared with the IL-$4^{+/+}$ and IL-$4^{+/-}$ groups, similar to findings in the original knockout strain (39). Following treatment with $HgCl_2$, all three groups had increases in total, IgG1, and IgG2a Ig levels (Table I); however, the IgG1 levels of IL-4-deficient mice remained substantially below the wild-type and heterozygous mice (52 ± 8 vs 1233 ± 218 μg/ml, $p < 0.001$, and 1561 ± 437 μg/ml, $p < 0.01$, respectively), and IgG2a levels were higher in the IL-$4^{-/-}$ group compared with wild-type mice (410 ± 102 vs 157 ± 34 μg/ml, $p = 0.01$).

Following $HgCl_2$ treatment, all groups had increased levels of ANoA ($p < 0.001$) and anti-chromatin Abs ($p < 0.05$) (Table II). Furthermore, similar levels of ANoA were observed with no significant differences in the percentage of animals positive for ANoA (83, 100, and 100% for +/+, +/−, and −/− mice, respectively) or in the mean ANoA titers (816 ± 314, 1298 ± 342, and 813 ± 151 for +/+, +/−, and −/− mice, respectively). Immunoprecipitation analysis also showed that the ANoA response in IL-$4^{-/-}$ mice was directed against fibrillarin (data not shown). No significant differences in anti-chromatin Ab levels were found among the $HgCl_2$-treated groups (1.051 ± 0.304, 1.752 ± 0.417, and 1.189 ± 0.334 for +/+, +/−, and −/− mice, respectively, $p > 0.05$).

Immunohistologic examination revealed that all $HgCl_2$-treated groups had increases in IgG and C3 deposits in glomeruli and splenic vessels compared with PBS controls (Table III). Moreover,

Table II. *Autoantibody levels in $HgCl_2$-treated IL-4-deficient mice*

Mice	Treatment	ANoA[a]		Anti-Chromatin Abs[b]	
		No. positive	Score	Prebleed	Postbleed
IL-4^{++}	PBS	0/7	0	0.000 ± 0.000	0.002 ± 0.002
IL-$4^{+/+}$	$HgCl_2$	10/12 (83)	816 ± 314	0.021 ± 0.017	1.051 ± 0.304
IL-$4^{+/-}$	PBS	0/6	0	0.002 ± 0.002	0.008 ± 0.008
IL-$4^{+/-}$	$HgCl_2$	9/9 (100)	1298 ± 342	0.008 ± 0.005	1.752 ± 0.417
IL-$4^{-/-}$	PBS	0/2	0	0.005 ± 0.002	0.007 ± 0.001
IL-$4^{-/-}$	$HgCl_2$	8/8 (100)	813 ± 151	0.005 ± 0.003	1.189 ± 0.334

[a] ANoA results are from sera obtained 4 wk after first $HgCl_2$ injection. All preimmunization sera were negative for ANoA. No. positive = number of mice with titers 1+/total number of animals; corresponding percentages of $HgCl_2$ treatment groups are indicated in parentheses. Scores are mean ± SE of the inverse titer that gave a 1+ ANoA pattern immunofluorescence score (0–4+ scale). For all mice, $p < 0.001$ when PBS vs $HgCl_2$ treatment groups compared. For comparisons between $HgCl_2$ treated mice, $p > 0.05$ for all combinations.
[b] Mean ± SE of anti-chromatin OD_{405} U. For postbleed PBS vs $HgCl_2$ treatment groups, $p \leq 0.001$ for IL-$4^{+/+}$ and IL-$4^{+/-}$, and $p = 0.04$ for IL-$4^{-/-}$ mice.

Table III. *Immunopathology of $HgCL_2$-treated IL-4-deficient mice*[a]

Mice	Treatment	Glomerular IgG[b]	Glomerular C3[b]	Spleen IgG[c]	Spleen C3[c]
IL-$4^{+/+}$	PBS	53 ± 53	53 ± 34	0	0
IL-$4^{+/+}$	$HgCl_2$	853 ± 164	293 ± 70	1.8 ± 0.37	0.96 ± 0.22
IL-$4^{+/-}$	PBS	240 ± 209	176 ± 39	0	0
IL-$4^{+/-}$	$HgCl_2$	1422 ± 142	702 ± 160	2.6 ± 0.24	1.7 ± 0.37
IL-$4^{-/-}$	PBS	0	0	0	0
IL-$4^{-/-}$	$HgCl_2$	869 ± 206	389 ± 98	1.0 ± 0.42	0.56 ± 0.26

[a] See Table I for number of animals in each group.
[b] Mean ± SE of the reciprocal titer of fluorescenated anti-mouse IgG and C3. $p < 0.05$ for $HgCl_2$-treated groups.
[c] Mean ± SE of immunofluorescence score (0–4+).

the increases were similar in all $HgCl_2$-treated groups, although IL-$4^{+/-}$ mice appeared to have slightly worse disease. This, however, did not reach statistical significance. Thus, IL-4-deficient mice appear to be not only susceptible to HgIA, but to have no decrease in disease severity.

$HgCl_2$ treatment of IFN-$\gamma^{-/-}$ mice

The role of IFN-γ (Th1-type responses) in HgIA was similarly studied using three groups of IFN-γ mice: wild-type (IFN-$\gamma^{+/+}$) and heterozygous (IFN-$\gamma^{+/-}$) or homozygous (IFN-$\gamma^{-/-}$) knockout mice. When total IgG and IgG1 and IgG2a isotypes were examined, there was variability in prebled and postbled levels among individual mice, possibly due in part to differences in background genes (Table IV). Nevertheless, higher IgG1 levels were seen in the $HgCl_2$- compared with PBS-treated groups for IFN-$\gamma^{+/+}$ and IFN-$\gamma^{+/-}$ mice (711 ± 87 vs 264 ± 32 μg/ml, and 1033 ± 117 vs 452 ± 54 μg/ml, $p < 0.02$). Between the IFN-$\gamma^{-/-}$ $HgCl_2$- and PBS-treated groups, however, there was no difference, despite comparable levels of IgG1 in $HgCl_2$-treated IFN-$\gamma^{-/-}$ (787 ± 137 μg/ml) and IFN-$\gamma^{+/+}$ mice (above). This was due to the higher IgG1 levels in the PBS-treated group (593 ± 114 μg/ml, $p > 0.05$) (Table IV). IgG2a levels were decreased in the $HgCl_2$-treated knockout groups (171 ± 46, 52 ± 14, and 21 ± 4 μg/ml for +/+, +/−, and −/− groups, respectively; $p < 0.02$ for +/+ vs −/− groups).

IFN-γ was found to have a profound effect on Ab responses to both nucleolar and chromatin Ags (Table V). Following $HgCl_2$ treatment, ANoA Abs were detected in 93% of IFN-$\gamma^{+/+}$, 75% of IFN-$\gamma^{+/-}$, and none of the IFN-$\gamma^{-/-}$ mice. The levels of ANoA ($=1^+$ intensity) correlated with the number of functional IFN-γ genes (773 ± 314, 73 ± 28, and 0 inverse titers for +/+, +/−, and −/− groups, respectively; $r = 0.47$, $p = 0.03$). A similar gene dose response was observed for anti-chromatin Ab production (1.027 ± 0.305, 0.147 ± 0.065, and 0.044 ± 0.019 for +/+, +/−, and −/− groups, respectively). Autoantibodies were not completely absent in IFN-$\gamma^{-/-}$ animals, however, as two of eight such mice injected with $HgCl_2$ had trace, but definite, ANoA at a serum dilution of 1/40.

IgG and C3 deposits in the kidney glomeruli and splenic vessels after $HgCl_2$ exposure were essentially absent in the IFN-$\gamma^{-/-}$ group and decreased in the IFN-$\gamma^{+/-}$ compared with wild-type mice ($p < 0.05$ for IFN-$\gamma^{+/+}$ vs IFN-$\gamma^{-/-}$ or IFN-$\gamma^{+/-}$ groups; Table VI). Thus, IFN-γ is required for the development of HgIA and appears to play a major role at the level of autoantibody production.

T-dependent NP-hapten response is decreased in IFN-$\gamma^{-/-}$ mice at low immunizing doses

The decreased response to two different autoantigens (nucleolar and chromatin) along with the normal immunopathology suggests that IFN-γ-deficient mice are unable to generate a significant autoantibody response. Indeed, the overall lack of response argues that self Ags may possess specific properties that differentiate them from foreign Ags. This possibility is supported by several studies showing essentially normal Ab responses of IFN-γ-deficient mice to nonself Ags and infectious agents, other than a shift away from IgG2a and IgG3 isotypes (47, 48). However, decreased Ab response has been observed in some cases (49), suggesting that an attenuated response may not be unique to self Ags. To examine this possibility, we compared the response of wild-type and IFN-$\gamma^{-/-}$ mice with different doses of the foreign T-dependent hapten, NP. The capacity for affinity maturation was also determined, since this is an important characteristic of some anti-nuclear Abs (50).

The IgM response to NP was similar in the wild-type and knockout mice for a given immunizing dose (Fig. 1, *A* and *B*). The IgG primary response for IFN-$\gamma^{-/-}$ mice immunized with high doses (100 μg) was slightly lower compared with IFN-$\gamma^{+/+}$ mice at day 9 (780 ± 138 vs 1179 ± 81 for the NP-20 substrate, $p < 0.05$);

Table IV. *Total IgG, IgG1, and IgG2a levels in $HgCl_2$-treated IFN-γ-deficient mice*

Mice	Treatment	No.	Total IgG[a] (μg/ml)		IgG1 (μg/ml)		IgG2a (μg/ml)	
			Prebleed	Postbleed	Prebleed	Postbleed	Prebleed	Postbleed
IFN-$\gamma^{+/+}$	PBS	5	2652 ± 340	5655 ± 747	231 ± 56	264 ± 32	38 ± 14	95 ± 28
IFN-$\gamma^{+/+}$	$HgCl_2$	15	1254 ± 153	4821 ± 540[b]	232 ± 40	711 ± 87[c]	13 ± 3	171 ± 46[c]
IFN-$\gamma^{+/-}$	PBS	5	828 ± 98	3293 ± 481	223 ± 33	452 ± 54	8 ± 3	83 ± 34
IFN-$\gamma^{+/-}$	$HgCl_2$	7	698 ± 108	2856 ± 449	169 ± 24	1033 ± 117[c]	34 ± 12	52 ± 14
IFN-$\gamma^{-/-}$	PBS	7	3210 ± 671	3099 ± 868	451 ± 122	593 ± 114	11 ± 2	10 ± 1
IFN-$\gamma^{-/-}$	$HgCl_2$	8	1035 ± 255	2350 ± 543	330 ± 73	787 ± 137	8 ± 1	21 ± 4[d]

[a] Ig levels are expressed as mean ± SE for the number of mice indicated. Pre- and postbleed sera were obtained prior to and after 4 wk of treatment. Serum Igs were quantiated by ELISA (see *Materials and Methods*).
[b] $p < 0.025$ for $HgCL_2$-treated IFN-$\gamma^{+/+}$ mice vs $HgCl_2$-treated IFN-$\gamma^{+/-}$ or IFN-$\gamma^{-/-}$ groups.
[c] $p < 0.02$ between $HgCl_2$ and PBS treatment groups.
[d] $p = 0.03$ for $HgCl_2$-treated IFN-$\gamma^{+/+}$ vs IFN-$\gamma^{-/-}$ groups.

Table V. *Autoantibody levels in $HgCl_2$-treated IFN-γ-deficient mice*

Mice	Treatment	No. Mice	ANoA[a]		Anti-Chromatin Abs[b]	
			No. positive (%)	Score	Prebleed	Postbleed
IFN-$\gamma^{+/+}$	PBS	5	0/5	0	0.009 ± 0.001	0.010 ± 0.001
IFN-$\gamma^{+/+}$	$HgCl_2$	15	14/15 (93)	773 ± 314	0.027 ± 0.007	1.027 ± 0.305
IFN-$\gamma^{+/-}$	PBS	7	0/7	0	0.037 ± 0.008	0.076 ± 0.036
IFN-$\gamma^{+/-}$	$HgCl_2$	8	6/8 (75)	73 ± 28	0.043 ± 0.009	0.147 ± 0.065
IFN-$\gamma^{-/-}$	PBS	7	0/7	0	0.002 ± 0.001	0.004 ± 0.001
IFN-$\gamma^{-/-}$	$HgCl_2$	8	0/8 (0)	0	0.010 ± 0.001	0.044 ± 0.019

[a] Only ANoA patterns included in table (the two IFN-$\gamma^{+/-}$ mice without ANoA had 2+ to 3+ diffuse homogenous ANAs). Results from sera obtained 4 wk after first $HgCl_2$ injection. All preimmunization sera were negative for ANA. No. positive = number of mice with titers 1+/total number of animals; corresponding percentages of $HgCl_2$-treatment groups are indicated in parentheses. Titers are mean ± SE of the inverse titer that gave a 1+ ANoA pattern immunofluorescence score (0–4+ scale). Three $HgCl_2$-treated mice, a IFN-$\gamma^{+/+}$ and two IFN-$\gamma^{-/-}$, had weak (< 1 + titers), but definite ANoA staining. For unpaired *t* tests of PBS vs $HgCL_2$ treatment groups, $p < 0.001$ for IFN-$\gamma^{+/+}$ and IFN-$\gamma^{+/-}$, but $p > 0.05$ for IFN-$\gamma^{-/-}$ mice. Among $HgCL_2$-treated groups, $p < 0.0005$ for IFN-$\gamma^{+/+}$ vs IFN-$\gamma^{+/-}$ vs IFN-$\gamma^{-/-}$mice, but $p > 0.05$ for IFN-$\gamma^{+/+}$ vs IFN-$\gamma^{+/-}$ mice.

[b] Mean ± SE of anti-chromatin OD_{405} U. Postbleed $HgCL_2$ treatment group comparisons (Mann-Whitney *U* test) were as follows: IFN-$\gamma^{+/+}$ vs IFN-$\gamma^{+/-}$ (p = 0.004) and IFN-$\gamma^{-/-}$ ($p = 0.002$); IFN-$\gamma^{+/-}$ ($p > 0.05$). For PBS vs $HgCl_2$ treatment groups; $p < 0.003$ for IFN-$\gamma^{+/+}$ mice, but $p > 0.05$ for IFN-$\gamma^{+/-}$ and IFN-$\gamma^{-/-}$ mice.

however, 15-day and secondary responses to NP were similar ($p > 0.05$; Fig. 1, *D* and *E*). Contrastingly, immunization with low doses of NP-hemocyanin (1 and 5 μg) resulted in lower secondary (28-day) IgG responses in IFN-$\gamma^{-/-}$ mice ($p < 0.05$ for 1 μg and 5 μg doses; Fig. 1, *D* and *E*).

In contrast to the above differences in IgG response to high and low doses of NP-hemocyanin, affinity maturation (NP-2.5/NP-20 ratio) of the IgG anti-NP response was similar for IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ mice for all three doses ($p > 0.05$; Fig. 1*F*). No difference was also observed in the affinity maturation of IgM Abs between the IFN-γ groups (+/+, +/−, and −/−) for the three doses of NP-hemocyanin ($p > 0.05$; Fig. 1*C*).

Discussion

The two major findings in this study are that HgIA is dependent on Th1-type, but not Th2-type cytokine responses, and that IFN-γ is important for optimal in vivo IgG Ab responses to low doses of Ag. Although the systemic autoimmunity induced by $HgCl_2$ has been considered to be a prototypic Th2-mediated disease, by using IL-4- and IFN-γ-deficient mice, we clearly show that this is not the case. While this seems paradoxical, given HgIA is a humoral immune mediated disease associated with increases in Th2-type cytokines and IgG isotype responses, nonetheless, other autoantibody-mediated diseases, including spontaneous lupus (36, 37, 51) and experimental myasthenia gravis (52), were also recently found to be IFN-γ dependent. Thus, without exception, humoral mediated autoimmune diseases have been found to be Th1 dependent, regardless of whether cytokine and IgG isotypes in diseased animals are of predominantly Th2 (this study) or Th1 (spontaneous lupus) type.

Table VI. *Immunopathology of $HgCl_2$-treated IFN-γ-deficient mice*

Mice[a]	Treatment	Glomerular IgG[b]	Glomerular C3[b]	Spleen IgG[c]	Spleen C3[c]
IFN-$\gamma^{+/+}$	PBS	0	32 ± 32	0	0
IFN-$\gamma^{+/+}$	$HgCl_2$	688 ± 122	392 ± 93	1.1 ± 0.49	0.44 ± 0.18
IFN-$\gamma^{+/-}$	PBS	46 ± 46	309 ± 66	0	0
IFN-$\gamma^{+/-}$	$HgCl_2$	120 ± 84	130 ± 77	0.25 ± 0.16	0.25 ± 0.16
IFN-$\gamma^{-/-}$	PBS	11 ± 11	74 ± 16	0	0
IFN-$\gamma^{-/-}$	$HgCl_2$	45 ± 26	15 ± 7	0.06 ± 0.06	0

[a] See Table V for number of mice in each group.

[b] Mean ± SE of the reciprocal titer of fluoresceinated anti-mouse IgG or C3 Ab. $p < 0.05$ for $HgCl_2$-treated IFN$^{+/+}$ vs IFN$^{+/-}$ or IFN$^{-/-}$ groups.

[c] Mean ± SE of immunofluorescence score (0–4+).

Based on these findings, we propose that the balance between Th1- and Th2-type responses does not directly contribute to autoimmune susceptibility. Indeed, the observations suggest that the findings in IFN-γ-deficient mice are not due to Th1/Th2 predominance per se, but to the requirement for IFN-γ to activate the immune system to respond to poorly antigenic determinants, including both self and nonself Ags. That IL-4$^{-/-}$ mice (Th1 predominance) did not show more severe disease than wild-type mice (Th2 predominance) is consistent with this possibility. Thus, the requirement for IFN-γ may be applied to both cellular (as has been described for the lymphocytic choriomeningitis virus model of insulin-dependent diabetes mellitus; Ref. 53) and humoral mediated (this study and Refs. 36, 37, and 52) autoimmune diseases. Moreover, as there appears to be a dose relationship between disease susceptibility and the number of functional IFN-γ genes, different levels of IFN-γ appear to directly contribute to disease susceptibility. This is supported by the recent report by Balomenos et al. (51), showing ~55% IFN-γ mRNA levels and attenuated disease in IFN-$\gamma^{+/-}$ MRL-*lpr* mice. Immunization with high doses of Ag and a strong adjuvant could be expected to bypass the requirement for IFN-γ. Indeed, several experimentally induced cell-mediated models of autoimmunity such as experimental allergic encephalomyelitis (54), experimental autoimmune uveoretinitis (55), and collagen-induced arthritis (56, 57) can be produced in IFN-γ-deficient mice. Similarly, a strong genetic predisposition may lower the susceptibility threshold sufficiently to overcome the requirement for IFN-γ, as suggested by the finding that IFN-γ-deficient nonobese diabetic mice develop insulin-dependent diabetes (58).

It can be argued that the induction of HgIA in IL-4 gene knockout mice may possibly be due in part to duplication or compensation of Th2 function. Although this cannot be ruled out, it would appear unlikely, since studies have established that IL-4$^{-/-}$ mice have little to no Th2 cell activity, as evidenced by the lack of expression of other Th2 cytokines in T cells and a skewed Ig isotype profile showing increased IgG2a and very low IgG1 and IgE serum levels (39, 59). Furthermore, in vitro studies have indicated that IL-4 is essential for the generation of the Th2 lineage (60, 61). Studies to look at GATA-3 expression and surface expression of CCR3 and the IFN-γR β_2 subunit might be used to confirm a decrease of Th2 cells. Moreover, recently developed conditional gene knockout techniques (62) that permit deletions of genes or cell types at specific times might provide a means to resolve this issue. Nevertheless, the IL-4 knockout would seem to

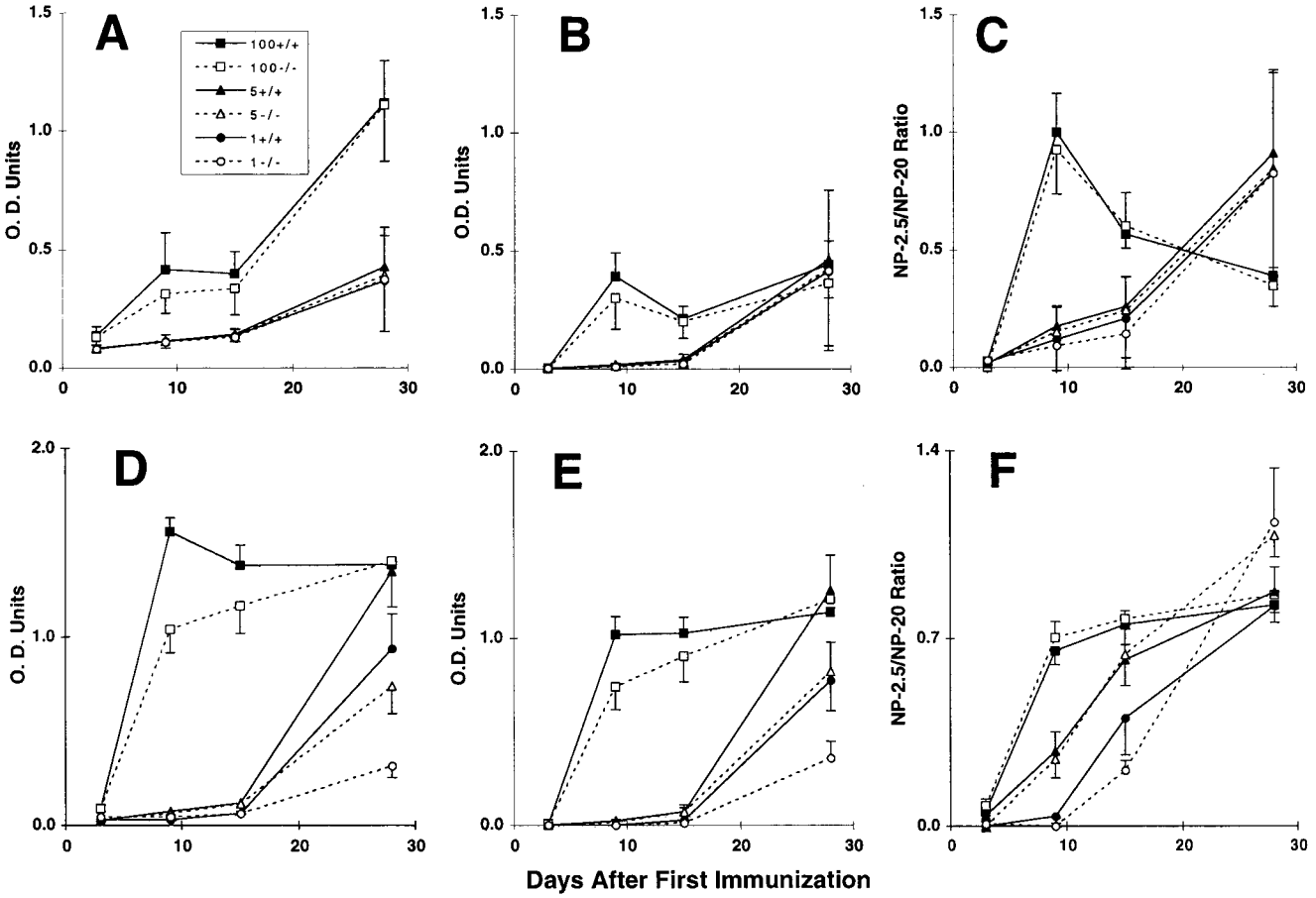

FIGURE 1. IgM and total Ig response of IFN-γ wild-type and knockout mice to NP. Mice were immunized i.p. with 100, 5, or 1 μg of NP conjugated to hemocyanin (20:1 ratio) in PBS on days 0 and 21. Sera were obtained on days 3, 9, 15, and 28, and analyzed by ELISA for hapten-specific IgM and total Ig (λ-chain) Abs to high (20:1, NP-20) and low (2.5:1, NP-2.5) density NP conjugated to BSA. *A*, IgM anti-NP-20; *B*, IgM anti-NP-2.5; *C*, IgM NP-2.5:NP-20 ratio; *D*, λ-chain anti-NP-20; *E*, λ-chain anti-NP-2.5; and *F*, λ-chain NP-2.5:NP-20 ratio.

represent the extreme end of the spectrum in terms of Th1/Th2 imbalance, and the induction of disease in IL-$4^{-/-}$ mice, but absence in IFN-$\gamma^{-/-}$ mice, strongly argues against a Th2 cell dependence of HgIA.

Recently, other exceptions to the Th1/Th2 paradigm in addition to this study and those discussed above for autoimmune responses have been reported, including studies of infections with intracellular pathogens, parasites and virus (63), and transplantation rejection (64, 65). Based on these findings, it is evident that the importance of a particular Th subset cannot be inferred by simple association and that the role of Th subtypes in immune processes is in most instances more complex than simple functional dichotomy. Thus, although the concept of Th1/Th2 has provided a useful and simple model for defining T cell regulation and function, delineation of the specific cytokines and cell types critical for the particular immune response is in most instances, such as in this study, more likely to be relevant than attempting to categorize responses as Th1 or Th2 (63).

Genetic (42) as well as Ig and TCR transgenic (66, 67) studies indicate susceptibility to autoimmune disease is a multistep process with several stages or checkpoints. The necessity for IFN-γ for autoantibody production defines a checkpoint at the initiation of the autoimmune response with the potential to discriminate self and nonself recognition. As such, the IFN-γ knockout autoimmune model may be useful for defining characteristics of self Ags and requirements for self Ag reactivity. Moreover, since IFN-γ is a highly pleiotropic cytokine with immune modulating, inflammation-promoting, and cell-regulatory properties, further definition of the specific autoimmune pathways that require IFN-γ may reveal specific therapeutic interventions that do not block effective responses to infectious agents.

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