Immunity, Vol. 14, 461–470, April, 2001, Copyright ©2001 by Cell Press

Type I Interferons Potently Enhance Humoral Immunity and Can Promote Isotype Switching by Stimulating Dendritic Cells In Vivo

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Summary

Type I interferons (IFN-I) are rapidly induced following infection and play a key role in nonspecific inhibition of virus replication. Here we have investigated the effects of IFN-I on the generation of antigen-specific antibody responses. The data show that IFN-I potently enhance the primary antibody response to a soluble protein, stimulating the production of all subclasses of IgG, and induce long-lived antibody production and immunological memory. In addition, endogenous production of IFN-I was shown to be essential for the adjuvant activity of CFA. Finally, IFN-I enhanced the antibody response and induced isotype switching when dendritic cells were the only cell type responding to IFN-I. The data reveal the potent adjuvant activity of IFN-I and their important role in linking innate and adaptive immunity.

Introduction

Signals generated after the recognition of infectious agents by cells of the innate immune system are thought to be important in triggering the antigen-specific adaptive immune response (Medzhitov and Janeway, 1997). In this regard, cytokines expressed in response to pathogen encounter may play a key role because of their potential to modify both the magnitude and the quality of the immune response elicited. Nevertheless, the immunomodulatory activity of some innate cytokines, including the type I interferons (IFN-I), remains largely unexplored.

IFN-I, the major species of which are IFN-α and -β, are expressed at low levels under normal physiological conditions but are induced to high levels by a number of stimuli, including viral or bacterial infection and exposure to double-stranded RNA (Belardelli et al., 1984; Kirchner, 1984; Bogdan, 2000). IFN-I are produced not only by immune cells but by virtually all cells, making them very effective in alerting the host to infection. They induce a variety of genes that can inhibit virus replication at several stages, conferring an anti-viral state on target cells (Kirchner, 1984), and are capable of activating both NK cells and macrophages (Belardelli, 1995; Biron et al., 1999; Bogdan, 2000). Hence, their role in the nonspecific resistance to virus spread is well recognized. Less clear is their importance in the generation of an antigen-specific adaptive immune response.

IFN-I have been shown to exert immunomodulatory effects in vitro. These include upregulation of MHC class I (Lindahl et al., 1976), promotion of Th1 responses by human but not mouse T cells (Brinkmann et al., 1993; Wenner et al., 1996), and enhancement of the maturation/activation state of dendritic cells (DC) generated from human peripheral blood or mouse bone marrow precursors (Luft et al., 1998; Paquette et al., 1998; Gallucci et al., 1999; Radvanyi et al., 1999; Santini et al., 2000). In addition, IFN-α was shown to act as a survival factor when added to type 2 DC precursors isolated from human blood, although it did not induce DC maturation. Interestingly, these cells produce very large quantities of IFN-α/β in response to viruses, implying that IFN-I are an autocrine survival factor for some DC precursors (Kadowaki et al., 2000). Thus, the ability of IFN-I to influence the activity of key immune cells suggests that these cytokines could serve as a link between the innate response to viruses and the adaptive immune response. However, IFN-I have also been shown to exert potent inhibitory effects on antibody production (Gisler et al., 1974) and T cell proliferation (Lindahl-Magnusson et al., 1972) in vitro, raising the question of whether these cytokines would act in a stimulatory or inhibitory manner in vivo.

Studies conducted by several groups in the 1970's aimed at determining the effects of systemic administration of IFN-I on the in vivo antibody response to sheep red blood cells provided conflicting results in this regard (Braun and Levy, 1972; Chester et al., 1973; Brodeur and Merigan, 1974, 1975; Strannegard et al., 1978; Vignaux et al., 1980). While some authors reported an inhibitory effect of IFN-I, others failed to see this inhibition and/or observed a modest stimulatory effect of IFN-I. The different results may have been related to the timing of injection of IFN-I, with the general trend being that treatment with IFN-I prior to immunization inhibited the response, whereas postimmunization inoculation of IFN-I enhanced antibody production. A similar influence of the timing of administration of IFN-I on the induction of DTH responses has also been reported (De Maeyer and De Maeyer-Guignard, 1980). Since expression of IFN-I after exposure to antigen was obviously more analogous to events occurring during a natural infection, the results together imply that virus-induced IFN-I could play a role in enhancing the immune response. However, these studies were limited by the lack of availability of reagents for showing definitively that the effects were mediated by IFN-I and for measuring the isotypes of antibodies produced. In addition, the use of sheep red

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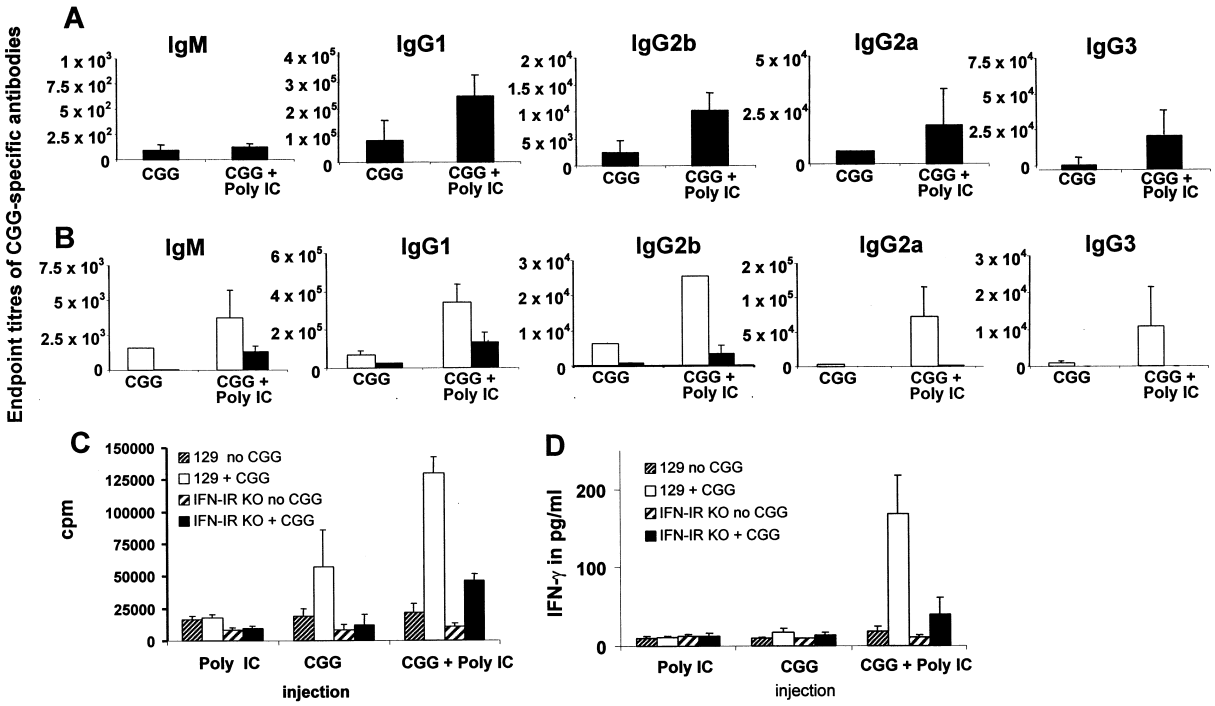

Figure 1. Poly IC Enhancement of the Primary Antibody and T Cell Response to CGG

B6 (A) or 129 and IFN-IR KO mice (B–D) were injected with poly IC, CGG, or CGG + poly IC as indicated. The sera and DrLNs were obtained 10 days later.

(A) Antibody response in B6 mice injected with CGG alone or CGG + poly IC.

(B) Antibody response in 129 (white bars) or IFN-IR KO (black bars) mice injected with CGG alone or CGG + poly IC. Antibody responses are expressed as the mean ± SD of endpoint titers.

(C) In vitro proliferation of T cells from 129 or IFN-IR KO mice primed by injection of poly IC, CGG, or CGG + poly IC. Cell suspensions from the DrLNs of 129 or IFN-IR KO mice were cultured ± CGG as indicated and pulsed with [^{3}H]thymidine on the fourth day of culture.

(D) IFN-γ secretion by CD4$^+$ T cells from 129 or IFN-IR KO mice primed by injection of poly IC, CGG, or CGG + poly IC. CD4$^+$ T cells purified from the DrLNs of immunized 129 or IFN-IR KO mice were cultured together with T-depleted spleen cells from nonimmunized syngeneic mice ± CGG as indicated. Supernatants were harvested on day 3 of culture and assayed for the presence of IFN-γ by ELISA. Data are derived from three to five mice per group.

blood cells, which induce strong immune responses on their own, may have restricted the ability to detect an enhancement of the response by IFN-I.

Other evidence that IFN-I may have immunostimulatory activity has come from a series of studies in transplantable tumor cell models (Belardelli and Gresser, 1996). Here it was found that rejection of IFN-resistant tumors was often enhanced by either coinjection of IFN-I or transfection of tumor cells with genes encoding IFN-α. Furthermore, this increased rejection appeared to be immune mediated and in some cases was associated with the induction of tumor-specific antibodies (Gresser et al., 1991).

Finally, there is evidence to suggest that IFN-I may influence the isotypes of antibodies secreted during an immune response (Finkelman et al., 1991). Hence, in mice stimulated to make a large polyclonal Ig response by injection of anti-IgD antibodies, treatment with IFN-α enhanced IgG2a levels while reducing the titers of IgG1 and IgGE antibodies. Whether IFN-I would have similar effects on an antigen-specific antibody response is unknown.

In the current study, we have examined directly how induction or administration of IFN-I at the site of immunization affects the generation of an immune response. The results show that IFN-I possess strong adjuvant activity, markedly enhancing the antibody response against a poorly immunogenic soluble protein. IFN-I augmented the production of all subclasses of IgG during the primary antibody response and induced both long-term antibody production and immunological memory after a single injection of soluble protein. In addition, evidence is presented that implicates DC as target cells for the adjuvanticity of IFN-I in vivo.

Results

Induction of Endogenous IFN-I Enhances the Primary Antibody Response

As an initial approach to determine whether IFN-I modulate antibody responses, we injected mice with polyinosinic:polycytidylic acid (poly IC), a synthetic double-stranded RNA, to induce production of IFN-I in vivo (Field et al., 1967). Thus, we immunized C57BL/6 (B6) mice by injecting chicken gamma globulin (CGG) in PBS sc and examined the effect of coinjecting poly IC. Ten days after immunization, the sera were assayed by ELISA for the presence of CGG-specific antibodies of various isotypes (Figure 1A). CGG alone was poorly immunogenic, and the response was largely restricted to

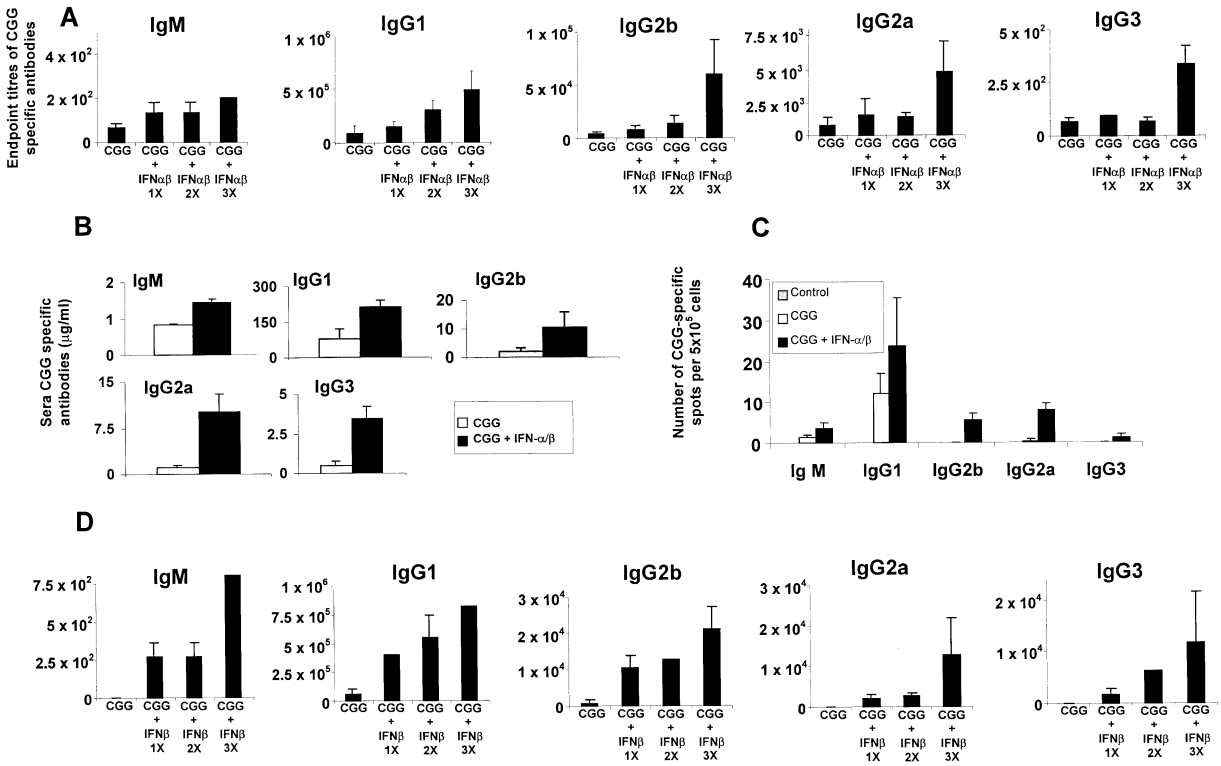

Figure 2. Enhancement of the Primary Antibody Response to CGG by Injection of IFN-I

(A and B) Antibody response in B6 mice immunized with CGG alone or immunized with CGG and treated with IFN-α/β.

(C) Frequency of cells secreting CGG-specific antibodies in DrLNs of B6 mice immunized with CGG alone or immunized with CGG and treated with IFN-α/β. Data are expressed as the number of cells secreting CGG-specific antibodies of particular isotypes per 5×10^5 total LN cells.

(D) Antibody response in B6 mice immunized with CGG alone or immunized with CGG and treated with IFN-β. All mice received a single injection of CGG. Those also receiving soluble IFN-α/β or IFN-β were injected with the respective IFN either only at the time of immunization (1×) or given additional injections of IFN 1 day later (2×) or 1 and 2 days later (3×). In (B) and (C), IFN-treated mice were given three injections of IFN-α/β. At day 10, mice were bled and their sera assayed for the presence of CGG-specific antibodies by ELISA. Antibody responses are expressed as the mean ± SD of endpoint titers, except in (B), where the approximate quantities of CGG-specific antibodies were determined by comparison with isotype standards (three mice per group).

antibodies of the IgG1 subclass; IgM, IgG2b, IgG2a, and IgG3 antibodies were detected at very low levels. Coinjection of poly IC stimulated a clear-cut increase in CGG-specific antibody titers, which applied to all subclasses of IgG (Figure 1A). This included 3-, 4.2-, 9-, and 8.4-fold increases in the titers of IgG1, IgG2b, IgG2a, and IgG3 antibodies, respectively. Poly IC similarly enhanced the antibody response to another soluble protein, ovalbumin (data not shown).

Although poly IC is known to be a potent inducer of IFN-I, it also induces other cytokines (Snapper et al., 1991; Roman et al., 1997). Therefore, it was important to determine whether the adjuvant activity of poly IC was in fact dependent on IFN-I. To do so, we compared the ability of poly IC to enhance the antibody response in mice lacking a functional receptor for IFN-I (IFN-IR KO mice, which were on a 129 background) and in control (129) mice. As in B6 mice, poly IC markedly enhanced the antibody response to CGG in control 129 mice (Figure 1B). In contrast, poly IC had a greatly reduced ability to do so in IFN-IR KO mice. Small increases in IgM, IgG1, and IgG2b titers were observed in IFN-IR KO mice, indicating that poly IC can enhance the production of these isotypes independently of IFN-I. However, most of the effect of poly IC was dependent on IFN-I, since the titers of these antibodies remained much lower in IFN-IR KO mice than in control mice. Furthermore, production of IgG2a and IgG3 anti-CGG antibodies was not stimulated at all by poly IC in IFN-IR KO mice. Taken together, these data show that induction of expression of IFN-I in the host stimulates a markedly increased antibody response to a soluble protein antigen, which includes antibodies of all IgG subclasses.

The effect of inducing IFN-I on T cell priming was also assessed. This was done initially by measuring the capacity of LN T cells to proliferate upon restimulation with CGG in vitro. Draining LNs (DrLNs) were removed 10 days after immunization and the resulting cell suspensions cultured in the presence or absence of CGG. Coinjection of poly IC with CGG into control (129) mice led to a much higher CGG-specific in vitro proliferative response than immunization with CGG alone (Figure 1C); pulsing with BrdU showed that most of the cells proliferating in vitro were $CD4^+$ (data not shown). The enhancement of T cell priming was partially independent of IFN-I, since poly IC treatment of IFN-IR KO mice also resulted in some increase in the in vitro proliferative response (Figure 1C). However, the proliferation of cells from

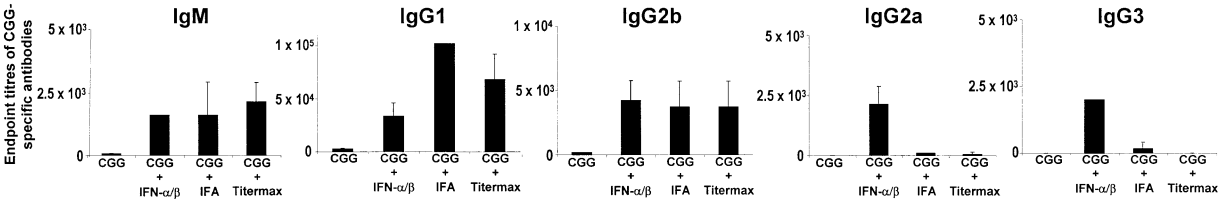

Figure 3. Comparison of Antibody Responses Enhanced by IFN-I and Oil-Based Adjuvants

B6 mice were immunized by sc injection of CGG, CGG + IFN-α/β, CGG emulsified in IFA, or CGG emulsified in TiterMax. All adjuvants were given together with CGG on day 0 and then alone on day 1 and 2. Ten days after challenge with antigen, sera were assayed for the presence of CGG-specific antibodies by ELISA. Results are expressed as the mean ± SD of endpoint titers (three mice per group).

CGG + poly IC–injected IFN-IR KO mice was much lower than that of cells from CGG + poly IC–injected control mice, indicating that IFN-I were in fact strongly enhancing the T cell response in vivo. This was also evident when cytokine production by in vitro restimulated $CD4^+$ T cells was examined (Figure 1D). Thus, while $CD4^+$ DrLN cells from mice immunized with CGG alone secreted little if any IFN-γ when stimulated by CGG in vitro, markedly higher amounts of IFN-γ were produced by $CD4^+$ cells from poly IC + CGG-injected mice. Importantly, poly IC augmented the priming of IFN-γ-secreting $CD4^+$ T cells to a much greater extent in control mice than in IFN-IR KO mice. In contrast, low amounts of IL-4 were secreted from $CD4^+$ cells in all groups that were not significantly different from each other (data not shown). Thus, induction of IFN-I in vivo enhances T cell priming, promoting the generation of IFN-γ-secreting $CD4^+$ T cells.

Stimulation of Primary Antibody Responses by Treatment with IFN-I

The effect of exogenous IFN-I on antibody responses was studied initially using a partially purified high titers preparation of murine IFN-I, which contained both IFN-α and IFN-β. B6 mice were injected sc with CGG alone or CGG + IFN-α/β (IFN 1×). In addition, separate groups of mice injected with CGG + IFN-α/β received a second sc injection of IFN-α/β alone 1 day later (IFN 2×) or sc injections of IFN-α/β both 1 and 2 days later (IFN 3×). As shown in Figures 2A and 2B, treatment of mice with IFN-α/β strongly enhanced the CGG-specific antibody response; the effect was most marked in mice receiving three injections of IFN-I and was apparent for all subclasses of IgG. CGG-specific IgE was not detectable in any group of mice (data not shown). Treatment with IFN-α/β similarly enhanced the antibody response in LPS-nonresponsive C3H/HeJ mice, discounting the possibility of contamination with endotoxin (data not shown). In addition to augmented serum antibody levels, higher numbers of antibody-secreting cells were detected in the DrLNs of IFN-α/β-treated mice (Figure 2C).

A similar experiment was performed using affinity-purified IFN-β (Figure 2D). In this case, a single injection of IFN-β was sufficient to enhance the primary antibody response, although, as for the partially purified IFN-α/β, the highest antibody titers were achieved after three injections of IFN-β. After one, two, or three injections of IFN-β, antibody titers were increased, respectively, 5-, 6-, and 8-fold for IgM, 6.4-, 8.5-, and 12.8-fold for IgG1, 13.3-, 16-, and 26.6-fold for IgG2b, 25.6-, 32-, and 153.6-fold for IgG2a, and 16.6-, 64-, and 117.3-fold for IgG3. Taken together with the experiment using partially purified IFN-α/β, these results clearly show that administration of IFN-I early during an immune response markedly increases the primary antibody response to a soluble protein antigen.

Comparison between IFN-I and Other Adjuvants

To evaluate further the efficiency of IFN-I as an adjuvant, we compared their capacity to enhance the primary antibody response with that of commercial adjuvants. Initial comparisons were made with two oil-based adjuvants, incomplete Freund's adjuvant (IFA) and Titermax (Figure 3). Although IFA and Titermax stimulated higher levels of IgG1 antibodies, IFN-I were equivalent to these adjuvants in ability to induce IgM and IgG2b antibodies and were far superior in increasing the production of IgG2a and IgG3 antibodies.

As a stricter test of adjuvant activity, IFN-I were compared to complete Freund's adjuvant (CFA). CFA has long been considered the "gold standard" for adjuvant activity in mice and is known to enhance the production of antibodies of all isotypes. Thus, we compared CGG-specific antibody titers, 10 days after immunization, in mice injected with CGG alone, CGG + CFA, or CGG + IFN-α/β; antibody responses were measured in B6 mice (Figure 4A) and in 129 mice (Figure 4B, white bars). As expected, antibody titers were higher in mice injected with CGG + IFN-α/β or CGG + CFA than in those immunized with CGG alone. Remarkably, the adjuvant activity of IFN-α/β compared favorably with that of CFA. In fact, although CFA induced higher titers of IgM antibodies (on the 129 background only), IFN-α/β stimulated the production of similar titers of IgG1 and IgG2b antibodies. Furthermore, IFN-α/β induced higher levels of IgG2a and, at least on the 129 background, IgG3 antibodies than CFA. These results showed, therefore, that IFN-α/β does indeed have powerful adjuvant activity. The effects are particularly significant when it is considered that the responses being compared were those to soluble protein + soluble IFN-α/β, which are likely cleared rapidly, and to the oily emulsion of CFA, which can persist at the site of injection for a long period of time.

Role of Endogenous IFN-I in the Adjuvant Activity of CFA and in Stimulation of the Response to Protein Alone

A notable difference between the adjuvant activities of CFA and IFA or Titermax is that only the former was able

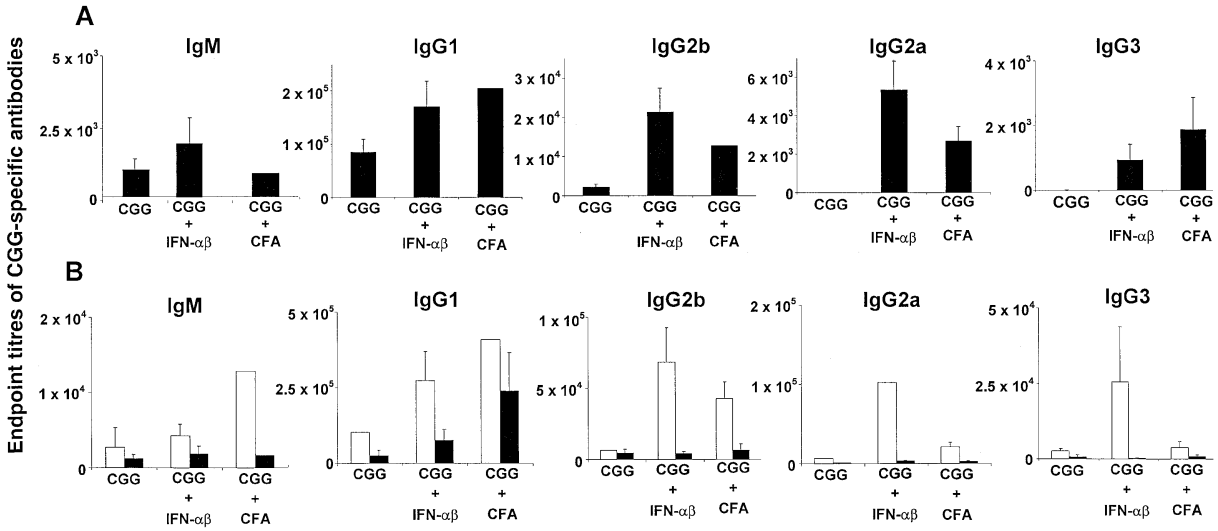

Figure 4. CFA Enhancement of Antibody Responses Is Similar to that Induced by Soluble IFN-α/β and Is Dependent on Endogenous IFN-I

(A) Comparison of antibody response in B6 mice injected with CGG alone, CGG + IFN-α/β, or CGG + CFA.

(B) Comparison of antibody response in WT 129 (white bars) or IFN-IR KO (black bars) mice injected with CGG alone, CGG + IFN-α/β, or CGG + CFA. Mice were immunized on day 0 by sc injection of CGG alone, CGG + IFN-α/β, or CGG emulsified in CFA. Mice received two more injections of IFN-α/β on day 1 and 2 postimmunization. At day 10, mice were bled and their sera assayed for the presence of CGG-specific antibodies by ELISA. Results are expressed as the mean ± SD of endpoint titers (three mice per group).

to induce significant titers of IgG2a or IgG3 antibodies. Since this is a property shared by IFN-I, it raised the question of whether the ability of CFA to do so was related to induction of endogenous IFN-I by this adjuvant. That IFN-I were induced by CFA seemed likely, given that a key constituent of CFA is heat-killed mycobacteria, and bacterial components such as CpG DNA are known to stimulate production of IFN-I (Sun et al., 1998b).

To test this hypothesis, we compared the antibody response to CGG in IFN-IR KO and control mice (Figure 4B). As expected, IFN-α/β was completely unable to enhance the response to CGG in IFN-IR KO mice. Importantly, the ability of CFA to promote the antibody response was also highly deficient in IFN-IR KO mice. Although CFA still induced high titers of IgG1 antibodies in IFN-IR KO mice, there was no longer any enhancement of IgM, IgG2b, IgG2a, or IgG3 antibodies compared to immunization with CGG alone. These results demonstrate an important role for IFN-I in the adjuvant activity of CFA.

It was also noted that the antibody response to CGG alone, although low, was consistently higher in control mice than in IFN-IR KO mice (Figures 1B and 4B). This suggested that the activity of low levels of "spontaneous" IFN-I might actually be important in the generation of an immune response to a soluble protein antigen in the absence of adjuvant. To address this possibility further, we injected sheep anti-IFN-α/β Ig into normal 129 mice to neutralize endogenous IFN-I and tested the effect on the antibody response to CGG alone. Mice were injected with anti-IFN-α/β antibodies twice: 4 days prior to priming and on the day of priming with CGG. Ten days after immunization, the antibody titers were determined and compared to mice injected with CGG alone. As shown in Table 1, mice treated with anti-IFN-α/β showed a dramatic decrease in their ability to mount an antibody response to CGG. The titers of all subclasses of IgG were reduced, while the production of IgM was not inhibited. Taken together with the data from the IFN-IR KO mice (Figures 1B and 4B), these results showed that background production of IFN-I can modify significantly the humoral response to a soluble protein.

Induction of Long-Term Antibody Production and Memory by IFN-I

Having shown that IFN-I enhanced the primary antibody response, it was of interest to determine whether this response was long lasting. Initially, we tested for long-term antibody production by assaying the sera of mice 6 months after a single injection of CGG or CGG + three injections of IFN-α/β (Figure 5A). Mice primed with CGG alone had extremely low levels of CGG-specific antibody after 6 months. In contrast, mice primed with CGG +

Table 1. Treatment with Anti-IFN-α/β Reduces Antibody Response to CGG Alone

Isotype	% Inhibition
IgM	−25
IgG1	76.25
IgG2b	94.45
IgG2a	77.08
IgG3	94.06

129 mice were either left untreated or received a prior injection of anti-IFN-α/β antibody 4 days before and on the day of immunization with CGG. CGG-specific antibody titer were determined by ELISA 10 days after immunization. Results are expressed as a percentage of inhibition of the antibody response to CGG by treatment with anti-IFN-α/β.

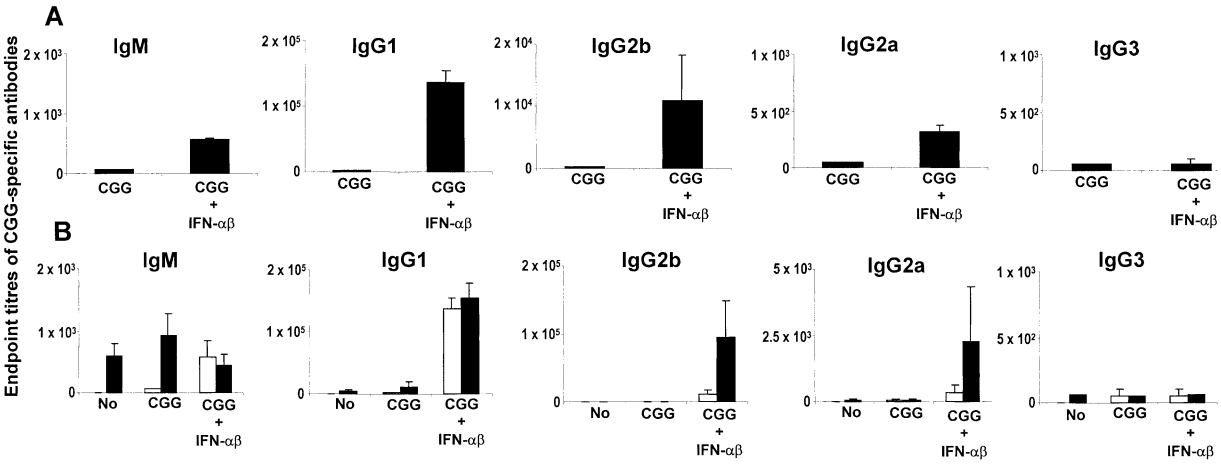

Figure 5. IFN-I Stimulate Long-Term Antibody Production and Immunological Memory

(A) Endpoint antibody titers in B6 mice immunized 6 months earlier by injection of CGG alone or CGG + IFN-α/β. B6 mice were immunized on day 0 by sc injection of either CGG alone or CGG + IFN-α/β. IFN-α/β treated mice received two more injections of IFN-α/β on day 1 and 2 postimmunization. Six months after immunization, mice were bled and their sera assayed for the presence of CGG-specific antibodies by ELISA.

(B) Antibody response 6 days after CGG challenge in naïve mice (No) and in mice immunized 6 months before with CGG alone or CGG + IFN-α/β. Immunized mice had been primed as in (A). CGG challenge was with CGG alone. Results represent antibody endpoint titers before (white bars) and 6 days after challenge (black bars) expressed as the mean ± SD of endpoint titers (three mice per group).

IFN-α/β still had significant titers of CGG-specific antibodies in their sera. With the exception of IgG3, for which titers were very low and not significantly different from those in mice primed with CGG alone, antibodies of all tested isotypes were present. Thus, injection of IFN-I during priming allowed for long-term antibody production.

It remains controversial whether long-term antibody production is maintained by long-lived plasma cells or by replenishment of antibody-producing cells from memory B cells. Nevertheless, there is strong evidence that the former do exist (Manz et al., 1997; Slifka et al., 1998), raising the theoretical possibility that the production of antibodies detected 6 months after injection with CGG + IFN-α/β could have persisted in the absence of memory B cells. Therefore, it was of interest to investigate whether memory was also induced by immunization in the presence of IFN-I. To do so, we examined the ability of mice primed 6 months earlier to mount a secondary response to CGG. Thus, mice that had been injected 6 months previously with CGG alone or CGG + three injections of IFN-α/β were reinjected with CGG alone. To minimize the contribution from a primary response to CGG, the secondary response was studied on day 6 after challenge and naive, nonprimed mice were used as controls. CGG-specific antibody titers were compared in the same mice before and after reinjection of CGG (Figure 5B). In mice primed 6 months earlier with CGG alone, the response to CGG challenge was indistinguishable from that in naive mice, indicating that there was no memory to CGG 6 months after priming with CGG alone. In marked contrast, however, mice primed 6 months previously with CGG + IFN-α/β did mount a rapid secondary response to CGG. The secondary response appeared, however, to be restricted to IgG2b and IgG2a antibody isotypes, despite the fact that high titers of IgG1 antibodies persisted in these mice. These results clearly showed that IFN-I promoted the generation of long-lived memory after a single injection of a soluble protein antigen.

Enhancement of the Antibody Response and Isotype Switching Occurs through Stimulation of Dendritic Cells by IFN-I

While IFN-I were clearly capable of markedly augmenting both the magnitude of the antibody response and switching to various IgG subclasses, their mechanism of action in vivo was unknown. However, since recent studies have shown that IFN-I can promote the maturation of human (Luft et al., 1998; Paquette et al., 1998; Radvanyi et al., 1999; Santini et al., 2000) and mouse (Gallucci et al., 1999) DC in vitro, one possibility was that IFN-I may also be acting on these cells in vivo. To test this hypothesis, we designed an adoptive transfer model in which DC were the only cells capable of responding to IFN-α/β. In these experiments, highly purified splenic DC from wild-type 129 mice or IFN-IR KO mice were incubated briefly with CGG and injected sc into IFN-IR KO recipients with or without IFN-α/β. Mice receiving CGG + DC + IFN-α/β were given two further injections of IFN-α/β as before. CGG-specific antibody titers were then measured on day 10 after immunization (Figure 6). As expected, IFN-α/β treatment of mice receiving CGG + IFN-IR KO DC did not enhance the antibody response, since no cells in these mice were able to respond to IFN-I. Conversely, injection of IFN-α/β into mice receiving CGG + wild-type DC induced an increase in antibody titers for all four IgG subclasses compared to injection of CGG + wild-type DC alone. Therefore, not only does stimulation of DC by IFN-I enhance the antibody response to coinjected protein, it is sufficient to induce isotype switching.

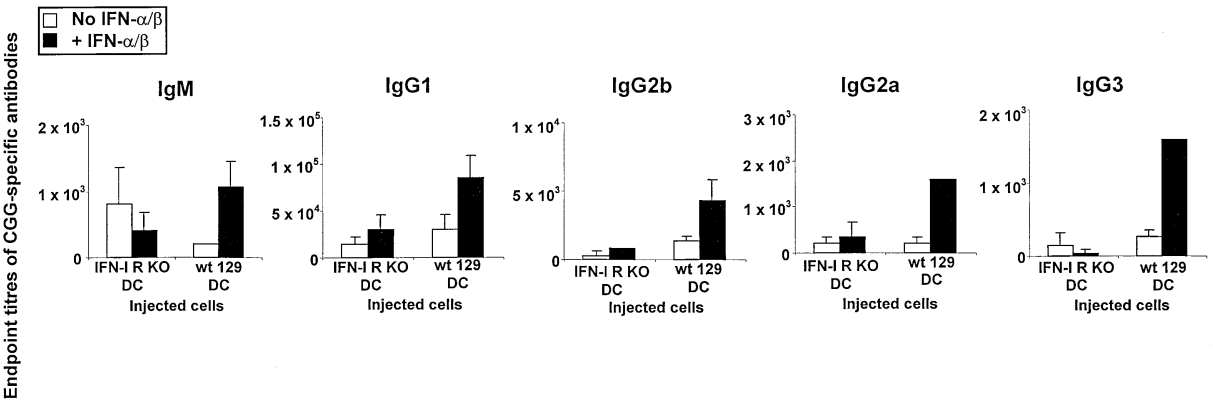

Figure 6. DC Responsive to IFN-I Are Sufficient for Enhancement of Antibody Production and Isotype Switching by IFN-I

Purified splenic DC were prepared from 129 mice (wt) or from IFN-IR KO mice, incubated briefly with CGG, and injected with (black bars) or without (white bars) IFN-α/β sc into IFN-IR KO mice. Mice injected with DC + CGG + IFN-α/β received two more injections of IFN-α/β on days 1 and 2 postimmunization. After 10 days, sera were assayed for the presence of CGG-specific antibodies by ELISA. Results are expressed as the mean ± SD of endpoint titers (three mice per group).

Discussion

Unlike purified protein antigens, infectious agents typically induce strong immune responses without the need for exogenous adjuvants. The implication is that nonantigen-specific signals induced by infection provide natural adjuvant activity, signaling the immune system to respond. We have shown here that such a signal can be provided by IFN-I. Thus, treatment of mice with IFN-α/β or induction of endogenous IFN-I by substances such as poly IC or CFA potently augmented the antibody response to a soluble protein. The adjuvant activity of soluble IFN-I was remarkably powerful, as exemplified by its ability to augment the primary antibody response to a similar extent as emulsification of the antigen in CFA. Furthermore, IFN-I were shown to enhance the production of antigen-specific antibodies of all subclasses of IgG and induced IgG2a and IgG3 antibodies far more effectively than widely used adjuvants such as IFA or Titermax.

As well as enhancing the primary response, IFN-I were also able to induce long-term antibody production after a single injection of soluble protein, which was remarkable considering the probable short half-life of both the antigen and the IFN-I in vivo. Thus, antibodies were still detectable 6 months after immunization in mice injected with CGG + IFN-α/β but not in mice injected with CGG alone. The issue of whether this reflected antibody production by long-lived plasma cells or by shorter lived plasma cells that were renewed from memory B cells was not addressed in this paper, but it is of interest that long-lived plasma cells have been detected after virus infection (Slifka et al., 1998). This raises the interesting possibility that IFN-I are involved in the generation of these cells.

In addition, mice immunized with CGG and treated with IFN-α/β exhibited a memory response upon challenge with CGG 6 months later, producing CGG-specific IgG2a and IgG2b antibodies within 6 days of injection of antigen alone; memory was not observed in mice immunized with CGG alone. Interestingly, CGG-specific IgG1 titers were not increased after challenge, despite the fact that these mice had high levels of IgG1 antibodies before challenge. These results are reminiscent of a previous report in which initial priming with tetanus toxoid in the presence of mouse hepatitis virus led to a secondary anti-tetanus response that included IgG2a but not IgG1 antibodies (Coutelier et al., 1991). The implication is that the virus-induced bias in the memory response may also have been mediated by IFN-I. The reason for the lack of secondary IgG1 response is unknown but could indicate that memory B cells expressing IgG1 are either not present or are driven to further isotype switching by interaction with memory T cells. The latter is worth considering, since there is considerable evidence that IgG1-producing B cells can switch to IgE production (Yoshida et al., 1990; Siebenkotten et al., 1992; Mandler et al., 1993).

Soluble IFN-I and CFA induced CGG-specific antibody responses that were similar in both magnitude and isotype distribution, which led us to investigate whether induction of IFN-I by CFA played a role in its adjuvant activity. Experiments in IFN-IR KO mice demonstrated that the capacity to respond to IFN-I was in fact essential for the adjuvant activity of CFA, particularly with regard to its ability to induce Ig class switching to IgG2b, IgG2a, and IgG3 subclasses of IgG. These findings raise the question of whether the induction of IFN-I might also be an important aspect of the activity of other adjuvants. Bacterial or CpG-containing DNA may be an interesting example in this regard, since it is able to act as an adjuvant in the promotion of IgG2a, IgG2b, and IgG3 responses (Roman et al., 1997; Sun et al., 1998a) and induces partial activation of T cells through a mechanism that is dependent on IFN-I (Sun et al., 1998b).

The ability of IFN-I to induce IgG2a antibodies is in accord with work published by Finkelman et al., where it was shown that administration of IFN-α enhanced IgG2a isotype switching in anti-IgD injected mice (Finkelman et al., 1991). Notably, IFN-α did not promote IgG2a production when added in vitro to LPS + IL-4 activated B cells, implying that class switching was me-

diated indirectly in vivo rather than through direct action of IFN-α on B cells. In the present study, we have identified DC as one target cell through which IFN-I can induce isotype switching, with the demonstration that IFN-α/β enhanced the production of not only IgG2a, but also IgG1, IgG2b, and IgG3 antibodies when DC were the only cell type able to respond directly to IFN-I in vivo.

Thus, although it cannot be ruled out that IFN-I exert effects on additional cell types that contribute to their adjuvant activity in normal mice, the adoptive transfer experiments clearly showed that stimulation of DC with IFN-I is sufficient to enhance antibody production and isotype switching; how this is accomplished is unknown. It is possible that DC mediate the adjuvant activity of IFN-I strictly through enhancing and polarizing T cell responses. As shown here, induction of IFN-I augments the priming of IFN-γ-producing $CD4^+$ T cells. While it seems likely that the elevated T cell response will contribute to the increased magnitude of the antibody response, it is uncertain whether IFN-γ production per se is important for the effects of IFN-I on the antibody response. In this regard, it has been shown that IFN-α-mediated enhancement of IgG2a isotype switching in anti-IgD-injected mice could not be blocked by anti-IFN-γ treatment (Finkelman et al., 1991). Furthermore, our preliminary results have shown that IFN-I can augment antibody responses and induce isotype switching in IFN-γR KO mice (A. L. B. et al., unpublished observations), implying that IFN-γ independence may also apply to the enhancement of antigen-specific humoral responses by IFN-I. Therefore, if DC mediate the adjuvant activity of IFN-I solely through their effects on T cells, mechanisms of T cell–induced isotype switching that are unrelated to IFN-γ secretion need to be considered.

An alternative possibility is that isotype switching was driven by non-T cells. The ability of *CD40L*$^{-/-}$ mice to produce IgG2a antibodies after virus infection suggests that typical T cell help may not be required for the isotype switch (Borrow et al., 1996). In this respect, IFN-stimulated DC might mediate their effects through direct interaction with B cells, since there is evidence to suggest that isotype switching can be regulated by DC–B cell contact (Wykes et al., 1998). Regardless of the mechanism, these results clearly show the importance of DC in linking the action of IFN-I to the adaptive immune response. While earlier studies showed an effect of IFN-I on the maturation of DC from precursors in vitro, here IFN-I was shown to modulate the ability of mature DC to alter the magnitude and the quality of an immune response in vivo.

Experimental Procedures

Mice

C57Bl/6 (B6) mice were purchased from Charles River-UK (Margate, Kent, UK) or from the SPF unit at the Institute for Animal Health (Compton, UK). C3H/HeJ mice were purchased from Harlan UK Ltd. (Blackthorn, UK). 129 SvEv (129) mice were purchased from the SPF unit at the Institute for Animal Health. 129 background mice deficient for type I IFN receptor function (IFN-IR KO) were originally purchased from B&K Universal (North Humberside, UK) and were maintained and bred in the SPF unit at the Institute for Animal Health.

Interferons

High titers IFN-α/β (2×10^7 U/mg of protein) was prepared in the C243-3 cell line following a method adapted from Tovey et al. (1974). Briefly, confluent cells were primed by the addition of 10 U/ml of IFN in MEM enriched with 10% FCS and 1 mM sodium butyrate. After 16 hr of culture at 37°C, C243-3 cells were infected by Newcastle disease virus (multiplicity of infection of 1) in MEM + 0.5% FCS + 5 mM theofylline. Eighteen hours postinfection, culture supernatant was collected and centrifuged at 1500 rpm for 10 min. The supernatant was adjusted to pH 2.0 and kept at 0°C for 6 days, before IFN titration. IFN was assayed by inhibition of the cytopathic effect of vesicular stomatitis virus on L cells in monolayer culture in Flacon microplates. These IFN preparations had the specific activity of 2×10^6 U/mg of protein after removal by centrifugation of contaminating protein precipitated during the treatment at pH 2.0 and dialysis against PBS. Units in the text are expressed as international mouse reference units. IFN was concentrated and partially purified by ammonium sulfate precipitations and dialysis against PBS. All IFN preparations were further subjected to dialysis for 24 hr at 4°C against 0.01 M percloric acid and then against PBS before testing them for any possible residual toxicity on a line of L1210 cells resistant to IFN. These partially purified IFN preparations had a titers of at least 2×10^7 U/mg of protein and were endotoxin free, as assessed by the *Limulus* amebocyte assay. They proved to be constituted of approximately 75% IFN-β and 25% IFN-α, as evaluated by neutralization assays using mAbs to IFNs, as described in detail elsewhere (Belardelli et al., 1987). High titers purified IFN-β (2×10^9 U/mg of protein) was prepared by affinity chromatography on a Sepharose column coupled with rat monoclonal antibodies to IFN-β (Kawade and Watanabe, 1987).

Protocols of Immunizations

All immunizations were done by sc injection of 100 μg of CGG (Stratech Scientific Ltd., Luton, UK). CGG was administrated in soluble form (in PBS) either when given alone or when mixed with 100 μg of poly IC (Sigma Chemical Co. Ltd., Dorset, UK), 10^5 U of IFN-α/β, or 10^5 U of purified IFN-β, as indicated. When injected with Titermax (CytRx Corporation, Norcross, GA), IFA (Sigma), or CFA (Sigma), an equal volume of CGG in PBS was emulsified with the adjuvant before injection. In some experiments, IFN-I, Titermax, or IFA were administered several times. In all cases, mice were injected sc at the site of the primary injection. Where indicated, the neutralizing sheep anti-IFN-α/β Ig was administrated ip in PBS 4 days before and at the time of injection with CGG. This antibody has been shown to neutralize the activity of all IFN-I naturally produced after virus infection of mouse cells but has no inhibitory activity against IFN-γ (Gresser et al., 1988). When testing for a memory response, mice were bled immediately prior to challenge with CGG to establish prechallenge antibody levels. The same mice were bled 6 days after CGG challenge.

Assay of Serum Antibody by ELISA

CGG (5 μg/ml in carbonate buffer [pH 9.6]) was coated overnight at RT in 96-well flexible plates (Falcon, Becton Dickinson, Oxford, UK). The plates were blocked with PBS containing 4% powdered milk for 1 hr at 37°C and then washed 3× in PBS-Tween (0.05%). Twelve-fold serial dilutions of sera in PBS-1% milk were added to the wells for 1 hr at RT. After three washes, biotinylated rat anti-mouse antibodies (anti-mouse IgM [R6-60.2], IgG1 [A85-1], IgG2a [R19-15], IgG2b [12-3], IgG3 [R40-82], or IgE [R35-72] [Becton Dickinson]) were added to the wells for 1 hr at RT. After three washes, streptavidin-HRP (Becton Dickinson) was added for 1 hr at RT. OPD tablets (Sigma) were used as peroxidase substrate. The reaction was stopped by addition of 50 μl 3 M HCl before the highest dilution of the highest titers serum rose above background. Optical densities were read at 492 nm on a SPECTRAmax (Molecular Devices, Sunnyvale, CA). Results are expressed as reciprocal endpoint titers, which were determined using an automated routine designed on Excel. Briefly, a threshold of positivity for OD values was calculated for each antibody isotype as the average + 3 SD of all dilutions from three control mouse sera (sera from either unmanipulated mice or mice treated with IFN-α/β or poly IC alone). The background level was very low at all dilutions (typically about 0.08) and did not vary significantly between experiments. For a given serum sample, the endpoint titers was determined as the first dilution below the threshold of positivity. Since endpoint titers are arbitrary units, the results

must be considered inside the same assays and cannot be directly compared between experiments. For this reason, all samples within in each experiment were assayed at the same time.

To determine the approximate concentrations of CGG-specific antibodies present in mouse sera, the ELISA was performed in a semiquantitative way by comparison to mouse Ig standards. CGG-specific antibodies were detected as described above, except that antibodies were revealed using isotype-specific polyclonal goat anti-mouse antibodies conjugated to alkaline phosphatase (AP) (all from Southern Biotechnology Associates Inc., Birmingham, AL). To establish standards, plates were coated with unlabeled isotype-specific polyclonal goat anti-mouse antibodies (5 μg/ml) (Southern Biotechnology). The plates were blocked as above and then purified mouse antibodies were added at known concentration (mouse Ig Standard Panel from Southern Biotechnology). After washes, the standards were revealed using isotype-specific goat anti-mouse antibodies conjugated to AP. p-NPP tablets (Sigma) were used as the AP substrate. The enzymatic reaction was stopped by adding 3 M NaOH. OD was read at 405 nm. Using SoftmaxPro (Molecular Devices, Sunnyvale, CA), we established standard curves for each isotype and calculated the amount of CGG-specific antibodies.

DC Preparation and Injection

DC were isolated from spleens using a method based on that described by Vremec et al. (1992). Briefly, spleens from 129 or IFN-IR KO mice were cut into small pieces and digested, with agitation, in RPMI containing 5% FCS, collagenase III (1 mg/ml, Lorne Laboratories, Reading, UK), and DNase I (0.6 mg/ml Sigma, St Louis, MO) for 5 min at 37°C followed by 15 min at RT. DC-enriched cell populations were obtained using Nycodenz (Life Technonology Paisley, UK) gradients. The low-density cell fraction was then labeled with anti-CD11c-FITC (Becton Dickinson, Oxford, UK) in PBS-EDTA-FCS for 20 min on ice. After washing, the cells were filtered (70 μm cell strainer, Falcon) and $CD11c^+$ cells were sorted on a MoFlow flow cytometer (Cytomation, Fort Collins, CO), with the resulting population being >98% $CD11c^+$. After two washes in PBS, purified DC were incubated in PBS alone or in PBS containing 100 μg CGG for 30 min at 37°C. Purified DC ($5–7 \times 10^5$), with or without CGG, were injected sc into IFN-IR KO mice ± 10^5 U IFN-α/β. Mice receiving CGG + DC + IFN-α/β were given additional sc injections of 10^5 U IFN-α/β 1 and 2 days later.

T Cell Proliferation and Cytokine Assays

DrLNs were cut into small pieces and digested in RPMI containing 5% FCS, collagenase III (1 mg/ml), and DNase I (0.6 mg/ml) for 20 min at RT with frequent mixing. Cell suspensions were then filtered (70 μm), washed, and centrifuged at 1500 rpm for 10 min. For proliferation assays, unseparated cells (5×10^5 per well) were cultured in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS [PAA Laboratories], 50 μM 2-ME [Sigma], 10 mM HEPES, 5% NCTC medium, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml gentamicin [all from Life Technologies]) in triplicate wells of 96-well plates ± CGG (20 μg/ well). On the fourth day of culture, wells were pulsed with 1 μCi [^{3}H]thymidine for 8 hr. Plates were then harvested and incorporated [^{3}H]thymidine measured using a MicroBeta TRILUX counter (Wallac, Turku, Finland). For cytokine assays, DrLN cells were incubated with anti-Class II (TIB120), anti-CD8 (3155), and anti-CD11b (M1/70) for 15 min on ice. After washing, $CD4^+$ T cells were purified by negative selection using sheep anti-rat IgG and anti-mouse IgG magnetic Dynabeads (Dynal, Oslo, Norway). Purified $CD4^+$ T cells (2×10^4) were cultured in complete medium in triplicate wells of 96-well plates with 5×10^5 T-depleted splenocytes from nontreated syngeneic mice. T-depleted splenocytes were prepared by incubating spleen cells for 45 min at 37°C with rat anti-mouse-Thy-1 antibody (T24) and guinea pig complement (VH BIO Ltd., Gosford, UK). Before culture, T-depleted splenocytes were preincubated ± CGG (20 μg/well) for 1 hr at 37°C and irradiated at 3000 rads. After 3 days of culture, supernatants were harvested and cytokines measured using the Quantikine M kits for mouse IFN-γ and IL-4 from R&D (Abingdon, Oxon, UK) as directed by the manufacturer.

CGG-Specific Elispot Assay

Multi-Screen-IP sterile Elispot plates (Millipore, Walford, UK) were coated overnight with CGG at 20 μg/ml in carbonate buffer (pH 9.6). After five washes in PBS, plates were blocked for 2 hr with 4% milk in PBS at 37°C and washed five times in PBS. Cell suspensions were prepared from DrLNs as described above, washed, centrifuged at 1500 rpm for 10 min, and resuspended in complete medium supplemented with 15% FCS. All samples were plated in triplicate at several different cell concentrations (from 2×10^4–5×10^5 cells/well). Following overnight culture at 37°C in 5% CO_2, plates were then extensively washed with PBS-Tween (0.05%). CGG-specific antibodies were revealed by incubating the wells with isotype-specific polyclonal goat anti-mouse antibodies conjugated to AP (Southern Biotechnology) for 2 hr at RT. After washes, BCIP (Sigma) diluted in 0.1 M Tris/HCl (pH 9.5); 10% diethanolamine; 0.1 M NaCl, 5 mM $MgCl_2$ at 1 mg/ml was used as the substrate for AP. The reaction was stopped by washing the plates with tap water. Spots were counted under a microscope.

Acknowledgments

We thank Persephone Borrow, Marion Berard, Peter Beverley, Lisa Hyland, and Steve Cose for critical review of the manuscript and helpful comments. This work was supported by The Edward Jenner Institute for Vaccine Research and by the European Community (contract Bio 04-CT98-0466). This is publication number 26 from the Edward Jenner Institute for Vaccine Research.

Received October 13, 2000; revised February 15, 2001.

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