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CpG Oligonucleotides Are Potent Adjuvants for the Activation of Autoreactive Encephalitogenic T Cells In Vivo

Benjamin M. Segal,1* John T. Chang,*† and Ethan M. Shevach2* 

The mechanism of action of microbial adjuvants in promoting the differentiation of autoimmune effector cells remains to be elucidated. We demonstrate that CpG-containing oligodeoxynucleotides (ODN) can completely substitute for heat-killed mycobacteria in the priming of encephalitogenic myelin-reactive T cells in vivo. The adjuvanticity of the CpG ODN was secondary to their direct ability to induce IL-12 or to act synergistically with endogenous IL-12 to promote Th1 differentiation and encephalitogenicity. T cells primed in the absence of CpG with Ag and IFA alone appeared to be in a transitional state and had not undergone differentiation along a conventional Th pathway. Unlike Th2 cells, they expressed low levels of the IL-12Rβ2 subunit and retained the ability to differentiate into encephalitogenic effectors when reactivated in vitro under Th1-polarizing conditions. These results support the use of CpG ODN as adjuvants but also suggest that they could potentially trigger autoimmune disease in a susceptible individual. The Journal of Immunology, 2000, 164: 5683–5688.

I t has been appreciated for many years that autoimmune diseases, including multiple sclerosis (MS),3 present as well as recur more frequently in the setting of infectious illness (1–9). Furthermore, to induce experimental autoimmune syndromes, it is often necessary to inoculate laboratory animals with the target autoantigen combined with microbial adjuvants. In the case of experimental allergic encephalomyelitis (EAE), an autoimmune demyelinating disease used as an animal model of MS, successful disease induction depends on the inclusion of heat-killed mycobacteria along with myelin proteins in the inoculum. In addition, many EAE protocols call for the coinjection of inactivated Bordetella pertussis (10). Administration of myelin Ags without microbial products (e.g., emulsified in IFA) fails to provoke disease and, in some instances, may actually result in a state of tolerance, whereby the recipient is resistant to subsequent attempts at disease induction (11, 12). It has been widely reported that lymph node (LN) cells and splenocytes from animals primed with Ags in IFA, as opposed to CFA, fail to mount proinflammatory responses. They do not secrete IFN-γ on challenge in vitro or mediate classic delayed type hypersensitivity reactions on challenge in vivo. Depending on the study, this has been attributed either to deletion/anergy of the Ag-specific T cells or to their differentiation along a Th2 pathway (13–16). On the other hand, under certain unusual circumstances, cells from animals primed repeatedly with neuroantigens in IFA have induced disease (17).

The mechanism of action of microbial adjuvants in promoting the differentiation of autoimmune effector T cells remains to be elucidated. The first aim of this study was to attempt to define the component(s) of whole microbial preparations that are responsible for their in vivo disease-promoting effects. We focused our efforts on the role of microbial DNA because it stimulates production of IL-12 (18–21), a cytokine that we and others have previously demonstrated to play a critical role in pathogenesis of EAE as well as other organ-specific autoimmune diseases (22–27). We demonstrate that CpG-containing oligodeoxynucleotides (ODN) that mediate some of the immunomodulatory functions of bacterial DNA could completely substitute for heat-killed mycobacteria and prime encephalitogenic myelin-reactive T cells in vivo. The second aim of the study was to further define the phenotype of autoreactive T cells that are primed in the absence of microbial adjuvants. We demonstrate that T cells from animals primed with myelin basic protein (MBP) in IFA fully retain the capacity to differentiate into encephalitogenic T cells when reactivated under conditions in vitro. Furthermore, our studies suggest that exposure to Ag in IFA partially activates T cells to differentiate along the Th1 pathway. Taken together, these observations have important implications both for our understanding of the role of environmental factors in the pathogenesis of autoimmune disease and for therapeutic strategies that use soluble Ags to tolerate or deviate the immune response.

Materials and Methods

Mice

Female SJL mice were obtained from the National Cancer Institute (Fredrick, MD) at 6–8 wk of age and housed in a pathogen-free facility. Mice were between 8 and 12 wk of age when experiments were initiated.

Peptides and oligonucleotides

MBP87–106 (VHVFFKNIVTPTPPPSQGK) was synthesized and purified by HPLC by the Laboratory of Molecular Structure, Peptide Synthesis Laboratory (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). ODN were purchased from Operon Technologies (Alameda, CA) and were phosphorothiolate modified to increase their resistance to nuclease degradation. The sequences were as follows: CpG ODN, ATAA TCAGGTTCCA AGCAAG; control (non-CpG) ODN, ATAA TAGAGCTTCAA GCAGAAG (28). The LPS content of the...
FIGURE 1. MBP-reactive T cells primed in the absence of microbial adjuvants expand but fail to differentiate into autoimmune effectors. CpG ODN are as effective as heat-killed mycobacteria in promoting encephalitogenicity. Female SJL mice were immunized with MBP<sub>87-106</sub> in PBS and an equal volume of CFA, IFA plus CpG ODN, or IFA alone. On day 10, draining LNs were resected and made into single-cell suspensions. A, Proliferative responses of LN cells after 96 h of culture. The data are representative of five experiments with similar results. B, IL-3 production. C, IFN-γ production by LN cells cultured for 72 h with or without MBP<sub>87-106</sub>. Cytokine production was generally below the limits of detection (12–24 pg/ml) in unstimulated cultures; measurable values were subtracted from the presented data. Mean values ± SD are shown for three experiments (p < 0.005). D, Ag-stimulated cells were harvested at 96 h, washed extensively, and injected i.p. into naive syngeneic recipients (5–8 mice/group). Recipient mice were monitored on a daily basis and rated on a 5-point scale as described in the text. In these experiments, all symptomatic mice progressed to a score of 2 or greater. Results are pooled from four experiments.

CpG ODN was <1 ng LPS/mg DNA in all instances, as measured by Limulus amebocyte assay (BioWhittaker, Walkersville, MD).

**Generation of MBP<sub>87-106</sub>-specific LN cells for cell transfer, mRNA measurements, and analysis of cytokine production**

Mice were immunized with MBP<sub>87-106</sub> (100 μg) emulsified in an equal volume of CFA, IFA, or IFA containing CpG ODN (60 μg) by s.c. injection at four sites over the flanks. In some experiments, mice were injected i.p. with a neutralizing mAb to IL-12, C17.8 (a gift of Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA), or normal rat IgG (Sigma, St. Louis, MO). Ten days later, draining LN (inguinal and axillary) were removed and processed as previously described (23). The lower limit of detection for each assay was 30 pg/ml. PCR fragments (50 ng) were labeled with [32P]dCTP using an oligolabeling kit (Pharmacia, Piscataway, NJ). Blots were prehybridized for 1 h at 42°C, followed by overnight hybridization with labeled probe at 42°C. Blots were then washed for 30 min in 2× SSC, 0.1% SDS buffer (room temperature) followed by 30 min in 0.1× SSC, 0.1% SDS buffer (55°C for IL-12Rβ2; 65°C for β-actin).

**Proliferation assays**

LN cells (4 × 10<sup>5</sup> in 0.2 ml) were cultured with various concentrations of Ag or medium alone in triplicate for 4 days in 96-well round-bottom plates (Costar, Cambridge, MA) Wells were pulsed for the final 16 h of culture with 1 μCi [3H]TdR (Amersham, Arlington, Heights, IL), and incorporated radioactivity was measured using a Betaplate scintillation counter (Wallac, Gaithersburg, MD).

**Northern blot analysis**

Total RNA was isolated from LN cell cultures using RNAzol RNA isolation solvent (Tel-Test, Friendswood, TX). Samples (10 μg total RNA per lane) were run on a 1.2% agarose gels containing MOPS buffer and formaldehyde and blotted onto a Hybond-N nylon membrane (Amersham). Membranes were baked for 2 h at 80°C and then probed for murine IL-12Rβ2 subunit or β-actin. Oligonucleotide probes were synthesized or purchased as previously described (29). The data are representative of five experiments with similar results. Values are the means ± SD are shown for three experiments (p < 0.005). Ag-stimulated cultures; measurable values were subtracted from the presented data. Mean values ± SD are shown for three experiments (p < 0.005). D, Ag-stimulated cells were harvested at 96 h, washed extensively, and injected i.p. into naive syngeneic recipients (5–8 mice/group). Recipient mice were monitored on a daily basis and rated on a 5-point scale as described in the text. In these experiments, all symptomatic mice progressed to a score of 2 or greater. Results are pooled from four experiments.

**Cytokine ELISA**

IL-2, IL-3, IL-4, IL-10, and IFN-γ were quantified using a sandwich ELISA technique based on noncompeting pairs of Abs as previously described (23). The lower limit of detection for each assay was 30 pg/ml or less.

**Results**

The adjuvant activity of CFA is duplicated by CpG ODN in IFA

Female SJL mice were immunized with MBP<sub>87-106</sub>, the immunodominant peptide of MBP that binds I-A<sup*d</sup>, emulsified with either IFA containing 1 mg/ml heat-killed Mycobacterium tuberculosis (CFA), IFA mixed with an immunostimulatory CpG-containing oligonucleotide (CpG ODN/IFA), or IFA alone. Draining LNs were removed 10 days later, dispersed into single-cell suspensions, and cultured with or without MBP<sub>87-106</sub> to measure Ag-specific lymphoproliferation and cytokine production. LN cells from all three treatment groups mounted significant proliferative responses (Fig. 1A) and produced similar quantities of IL-3 (Fig. 1B). IL-2 was produced in low amounts by all groups (<100 pg/ml). Therefore, we concluded that MBP-reactive T cells were neither deleted nor anergized as a consequence of being primed in the absence of microbial adjuvants. Indeed, the fact that we were able to detect...
The adjuvant effects of CpG-ODN are IL-12 dependent

We next used cytokine gene knockout mice to determine the relative importance of IFN-γ and IL-12 in the development of encephalitogenic T cells that were induced after priming with MBP/IFA/CpG ODN. We have previously demonstrated that IL-12-deficient (−/−) C57BL/6 mice are resistant to EAE induced by immunization with bovine MBP in CFA, whereas IFN-γ−/− mice are highly susceptible. Disease was suppressed in IFN-γ−/− mice by neutralization of IL-12 (24). These studies demonstrated that encephalitogenicity is induced by an IL-12-dependent, IFN-γ-independent pathway and that IFN-γ paradoxically suppresses EAE at some point in the pathogenic process.

In keeping with these earlier results, IL-12−/− C57BL/6 mice were resistant to EAE induction after immunization with MBP in CpG ODN/IFA. Furthermore, IFN-γ−/− mice were equally susceptible to EAE induced using either MBP in CFA or CpG ODN/IFA but did not develop disease when immunized with MBP in IFA alone (Fig. 2). These results suggest that the adjuvanticity of both intact mycobacteria and CpG-ODN is secondary to their ability to directly induce IL-12 or that they act synergistically with endogenous IL-12 to promote Th1 differentiation and encephalitogenicity. Furthermore, both microbial products achieve their adjuvant effects in the absence of IFN-γ.

We also examined whether the CpG ODN must be directly associated with MBP in the adjuvant emulsion for successful disease induction. IFN-γ−/− C57BL/6 mice were primed with bovine MBP in IFA s.c. and simultaneously injected with CpG ODN i.p., i.v., or in the foot pad. Whereas 100% of mice developed severe EAE when MBP87−106 and CpG ODN were combined in a single emulsion, they remained asymptomatic when MBP87−106 in IFA alone (Fig. 2). This result suggests that CpG ODN cannot mediate their adjuvant effects at a distance and that the same APC must present MBP and produce CpG ODN-induced factors (such as IL-12), or neighboring APCs must perform these functions in tandem.

Ag in IFA partially primes Th1 effector cells

MBP-reactive LN cells from IFA-primed mice neither secreted detectable quantities of IFN-γ in vitro nor transferred disease into naive syngeneic recipients (Fig. 1, C and D). Because it has recently been reported that foreign Ag-reactive T cells primed using CFA differentiate into Th1 effectors, whereas T cells of the same specificities primed using IFA default to a Th2 lineage (13), we tested whether T cells from donors immunized with MBP87−106 in IFA produce IL-4 and IL-10 on in vitro challenge. We were unable to detect either cytokine in the supernatants of any of the cultures in multiple experiments (data not shown).

An alternative possibility was that MBP-reactive T cells primed with IFA alone were in an intermediate stage of development that would allow them to mature into Th1 encephalitogenic effector cells after reactivation under Th1 polarizing conditions. Expression of the β2 subunit of the IL-12 receptor (IL-12Rβ2) is a critical step in Th1 differentiation (30). Furthermore, we have found that the ability of myelin protein-specific CD4+ T cells to induce EAE correlates with their ability to up-regulate IL-12Rβ2 on antigen stimulation (29). We therefore compared IL-12Rβ2 expression in LN cells from animals immunized with MBP87−106/IFA or MBP87−106/CpG ODN/IFA after restimulation with Ag in vitro. T cells from animals primed with Ag in CpG ODN/IFA significantly up-regulated IL-12Rβ2 mRNA upon in vitro challenge with Ag alone. IL-12Rβ2 mRNA expression was dependent on the presence of endogenous IL-12 because it was abrogated by the addition of anti-IL-12. By contrast, IL-12Rβ2 mRNA was only modestly induced by the reactivation of T cells that had been primed with MBP87−106 in IFA only. However, when recombinant IL-12 was added, these T cells up-regulated IL-12Rβ2 mRNA expression and secreted IFN-γ to levels comparable with those of MBP-reactive T cells that had been exposed to CpG ODN in vivo (Fig. 3, A and B).

More importantly, the MBP87−106/IFA primed LN cells were able to transfer moderate-severe EAE in 100% of naive syngeneic recipients after stimulation with IL-12 (Fig. 3C).

Induction of IL-12Rβ2 expression is critically dependent on the presence of IL-12 as T cells from IL-12−/− mice do not express the IL-12Rβ2 after stimulation with specific Ag or polyclonal stimulation with anti-CD3 (Ref. 29 and our unpublished data). The
presence of low, but detectable, levels of IL-12Rβ2 on T cells from mice primed with MBP/IFA (Fig. 3, lane 5) raised the possibility that immunization with Ag in IFA stimulated a low level of IL-12 production, sufficient to induce modest IL-12Rβ2 expression by the responder T cells but not sufficient to fully drive their differentiation along a Th1 lineage. To test this hypothesis, we immunized SJL mice with MBP87–106 in IFA and simultaneously injected either a neutralizing Ab against IL-12 or an isotype-matched control Ab. Ten days later, draining LN were removed and stimulated with MBP87–106 and recombinant IL-12. Whereas MBP-reactive LN cells from control Ab-treated donors secreted IFN-γ and transferred EAE to naive recipients after reactivation with IL-12, LN cells from anti-IL-12-treated donors, that were restimulated in the presence of IL-12, failed to produce IFN-γ and were unable to induce EAE on passive transfer (Fig. 4). LN cells from anti-IL-12-treated donors had not been deviated to a Th2 phenotype.

**FIGURE 3.** MBP-reactive LN cells from IFA-immunized mice retain the capacity to up-regulate IL-12Rβ2 chain and to differentiate into Th1 pathogenic effector cells after reactivation in the presence of IL-12. Draining LN were removed from mice immunized with MBP87–106 (in either IFA or IFA plus CpG ODN) as described in Fig. 2. A, LN cells were cultured with or without Ag. Recombinant murine IL-12 (20 ng/ml) or a neutralizing mAb against IL-12 (aIL-12) (C17.8, 10 μg/ml) were added to some wells as indicated in the figure. At 72 h, cells were harvested, and RNA was isolated. Northern blot analysis was performed using probes specific for murine IL-12Rβ2 chain and, subsequently, for β-actin. Data are representative of three experiments with similar results. B, Supernatants from some of the cultures described in A were collected at 72 h for IFN-γ quantification by sandwich ELISA. The results represent the difference between cytokine production in the presence or absence of Ag, respectively. Means ± SD of three experiments are shown (*, p < 0.005). C, LN cells from donors immunized with MBP87–106 in IFA were challenged with Ag in the presence (¡squlf) or absence (C) of recombinant IL-12 (20 ng/ml), harvested at 96 h, washed extensively, and injected i.p. into naive syngeneic recipients (60 × 10⁶ cells/mouse). Recipients were monitored on a daily basis and rated for clinical signs as described in Fig. 3C. The results shown are pooled from two experiments with five to seven mice per group.

**FIGURE 4.** Exposure to endogenous IL-12 during priming with MBP87–106 in IFA is required for differentiation to the pre-Th1 phenotype. SJL mice (20–30/group) were immunized with MBP87–106 in IFA s.c. and injected i.p. with either rat IgG or a neutralizing mAb to IL-12 on days 0 (1 mg/mouse), 3 (0.5 mg), and 6 (0.5 mg). LN cells were harvested on day 10 and cultured with MBP87–106 and IL-12 (20 ng/ml). A, Supernatants were collected at 72 h for IFN-γ quantification by sandwich ELISA. The results represent the difference between cytokine production in the presence or absence of Ag, respectively. Means ± SD of three experiments are shown (*, p < 0.005). B, LN cells were collected at 96 h, washed, and injected i.p. into naive syngeneic recipients (60 × 10⁶ cells/mouse). Recipients were monitored on a daily basis and rated for clinical signs as described in Fig. 3B. The results shown are pooled from two experiments with five to seven mice per group.
phenotype because they failed to produce IL-4 or IL-10 (data not shown). We conclude from these studies that small quantities of IL-12, produced either constitutively or in response to immunization, play a critical role in shaping the phenotype of MBP-reactive T cells primed with Ag in IFA alone; immunization by this route does not appear to result in the differentiation of autoreactive cells into either Th2 or Th1 (31) cells but appears to partially drive them along the Th1 pathway as demonstrated by their ability to up-regulate IL-12Rβ2 and differentiate into Th1 autoimmune effectors when reactivated in vitro in the presence of IL-12.

Discussion

The nature of individual Ag-specific immune responses is determined by a collaboration between the innate and adaptive immune systems. A variety of nonspecific signals delivered by live microbes and tumor cells in physiological settings and by adjuvants in experimental settings induce functional changes in dendritic cells and macrophages, the so-called “professional” APCs. This, in turn, influences the path subsequently taken by T cells activated in the same microenvironment. For example, a number of microbial preparations, such as heat-killed M. tuberculosis, stimulate IL-12 production by APCs. T cells activated by those APCs are thereby biased toward a Th1 lineage. Normally, this type of cross-talk is beneficial to the host; Th1 responses are most effective in eradicating mycobacterial infections (32). However, when the innate response becomes subverted to guide the development of autoreactive T cells, organ-specific autoimmune diseases may result. In this paper, we demonstrate that IFA containing a 20-base-long ODN with two unmethylated CpG dinucleotides is as effective an adjuvant as CFA in promoting the development of encephalitogenic T cells in vivo. These findings confirm and extend earlier reports of induction of potent Th1 responses to foreign Ags using a similar protocol with CpG ODN in IFA (28, 33, 34).

There is a wealth of circumstantial evidence supporting the conclusion that the major, if not the only, role of microbial adjuvants in EAE is to induce IL-12 production by APC during a crucial stage in the development of myelin-reactive T cells. We, as well as others, have established the importance of IL-12 in the pathogenesis of EAE (22–26). Furthermore, the disease-promoting actions of both CpG ODN/IFA and CFA correlate with their capacity to induce autoreactive Th1 differentiation, and neither adjuvant is effective in the absence of IL-12. Nonetheless, it is still possible that CpG ODNs stimulate APCs to produce other soluble factors such as type I IFNs or IL-18 (34–37) and/or induce the expression of cell surface molecules such as MHC class II or CD80/CD86 (38) that act synergistically with IL-12 during autoimmune pathogenesis. It has also been recently reported that CpG ODNs provide potent growth and maturation signals for dendritic cells (39, 40), have direct effects on NK cells and B cells (41–43), and may have APC-independent effects on T cells (41, 44). The experimental model described in this study should prove to be very useful in determining which of the pleiotropic effects of CpG are most important in the activation of autoreactive T cells.

For a control group in all of our studies, we immunized animals with Ag in IFA alone. IFA contains only mineral oil, which functions as a local Ag depot. T cells isolated from the draining LN of IFA immunized animals mounted as strong an Ag-specific lymphoproliferative response as T cells isolated from CFA-primed mice. Similar results were recently reported by other groups (13, 33). By contrast, several earlier studies found that immunization with Ags in the absence of microbial adjuvants lead to deletion, anergy, or active suppression of the targeted T cell population (14, 16, 45–49). In most of the latter studies, Ags were administered in relatively large quantities and delivered by alternate routes when used as a tolerogen in IFA as opposed to an immunogen in CFA. The paradoxical outcomes may have resulted from the targeting of different classes of APCs in the spleen and LN, respectively.

Chu et al. (28) demonstrated that immunization of mice with Ag in IFA, as opposed to CFA or CpG ODN/IFA, resulted in the production of comparable amounts of total Ag-specific IgG but failed to generate IFN-γ producing LN cells (28). These results and others (13, 15) have fostered the concept that T cells primed by Ag in CFA exclusively differentiate along a Th1 pathway, whereas those primed by Ag in IFA default to a Th2 lineage. In many of these studies, a Th2 response is defined by the expansion of IL-5-producing effector cells in the setting of a stable or diminished IFN-γ-producing population, and the induction of specific IgG1 but not IgG2a Abs. Our results strongly suggest that the concept that immunization with Ag in IFA results in a polar Th2 response is an oversimplification. T cells harvested from the draining LNs of MBP 87–106/IFA-primed mice appear to be in a transitional state and have yet to undergo terminal differentiation along a conventional Th pathway. After challenge in vitro, these cells do not produce detectable levels of IFN-γ, IL-4, or IL-10. Unlike Th2 cells, they express IL-12Rβ2, but at a level that is considerably lower than that expressed by typical Th1 cells primed with CpG ODN or CFA. Nevertheless, the level is sufficient to permit Th1 autoimmune effector differentiation after exposure to high doses of exogenous IL-12 in vitro. The capacity of anti-IL-12 to prevent the development of this unique population of cells after immunization with Ag in IFA contradicts the view that they are Th2 precursors because IL-12 antagonizes, rather than promotes, Th2 differentiation (50).

One possible explanation for the differences between our results and those of Yip et al. (13) is that we have studied the response to an autoantigen, whereas they studied responses to foreign Ags. Autoreactive T cells that escape negative selection in the thymus tend to bear TCRs with a relatively low affinity (51). It is possible that the foreign Ag-reactive T cells which bear higher affinity TCR are more likely to produce IL-4 during in vivo priming in the absence of microbial adjuvants and, consequently, to commit to a Th2 phenotype. If lower affinity autoreactive T cells fail to secrete IL-4, they may maintain IL-12 receptor expression (although at a relatively low level) and subsequently be able to differentiate into Th1 cells when reactivated under polarizing conditions. Alternatively, immunization with Ag in IFA may result in a mixed Th response with partial activation of Th1 cells and more complete activation of Th2 cells irrespective of the nature of the Ag used as an immunogen.

Myelin-reactive T cells exist in healthy individuals (52) and remain a potential reservoir of pathogenic effectors which, when appropriately stimulated, could precipitate an autoimmune state. We have previously characterized a population of T cells in B10.S mice after immunization with MBP in CFA (23, 29). These cells closely resemble the population of T cells generated by immunization of SJL mice with MBP in IFA. Both populations fail to produce IFN-γ in vitro when restimulated with Ag, fail to up-regulate their IL-12Rβ2 chain, and fail to transfer EAE. Moreover, after exposure to IL-12, both differentiate into pathogenic Th1 effector cells. Because some myelin Ags are expressed in peripheral sites (53), it is possible that in healthy individuals similar “benign” populations of autoreactive effector cells are activated in a noninflammatory setting at some point during their life spans. If this occurs, they would acquire the characteristics of memory cells and thereby resemble the “pre-Th1” cells we have described. Our data suggest that such T cells may be capable of fully differentiating into Th1 effectors on reactivation in an inflammatory milieu. Hence, our findings may explain, in part, the association between autoimmune episodes and infectious illnesses/vaccinations (1–9). They also raise a cautionary note regarding the use of DNA vaccinations with vectors containing immunostimulatory CpG motifs due...
References


