CpG ODN Enhances Immunization Effects of Hepatitis B Vaccine in Aged Mice

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Oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides in contexts of unique sequence (CpG motifs) is active as adjuvant in induction of cellular and humoral immune responses in young mice. To date, there are only limited reports about effect of CpG ODN on immune responses against hepatitis B (HB) infection in aged mice. Our studies demonstrated there were significant increases in secreting of total anti-HB IgG, IgG1 and IgG2a, as well as of IL-12 and IFN-γ, when CpG ODNs were injected together with hepatitis B antigen in aged mice. Moreover, CpG ODN could stimulate proliferation of spleen lymphocytes in a dose-dependent manner. Taken together, the results we obtained indicate that the adding of CpG ODN into the vaccine antigen might be useful in development of more effective vaccination for inducing anti-HB virus responses in the elderly. *Cellular & Molecular Immunology*. 2004;1(2):148-152.

Key Words: CpG oligodeoxynucleotide, aged mice, adjuvant, Vaccination

Introduction

Aged individuals commonly exhibit deficiencies in their ability to mount protective immune responses (1). Attempts to immunize elderly humans or aged experimental animals against infectious agents or bacterial toxins, often resulted in failure of eliciting a protective immune response (1, 2). While the reasons for such failure have not been fully resolved, a number of cellular and molecular changes are appreciated to occur during the normal aging process that could mediate the functional deficiencies responsible for predisposing elderly individuals not able to elicit in humoral and cellular immune responses (3). It is important to pursue in an immunostimulator to improve the responsiveness of aged individuals to primary immunization. One strategy would be to develop vaccines containing suitable adjuvants to initiate and elicit of protective immunity. Presently, the only adjuvant that has gained wide acceptance for use in human vaccines is the aluminum-based mineral salts (4). Although alum is inexpensive, stable, easy to formulate, exhibiting low toxicity and has a good safety record, its adjuvant properties are only minimally effective in the elderly when compared to the responses elicited by the same vaccine preparations used in young recipients. While several new adjuvants have been evaluated experimentally in animal models, studies have demonstrated that, these adjuvant formulations (with the possible exception of MF59) have been unable to provide the needed effects in humans (5-8). Recently, many studies showed prokaryotic DNA containing unmethylated CpG motifs could stimulate the immune system and induce the secretion of immunoglobulins and kinds of cytokines. Since several studies have now shown that CpG oligodeoxynucleotides (ODNs) act as effective adjuvants, capable of promoting antigen-specific immune responses in young experimental animals, vaccinated with foreign proteins (9-11). To date, there have been no studies about effect of CpG ODN on the humoral and cellular immune responses in aged mice. Our studies suggested that the development of vaccines containing CpG ODN as adjuvant might prove useful for increasing the success rate of vaccination in the elderly.

Materials and Methods

Reagents

Hepatitis B surface antigen (HBsAg) (10 mg/L) was produced by the Beijing Tiantan Biologic Product CO., LTD. Anti-IL-12 antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG were purchased from Huamei Biologic Engineering Company.

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Abbreviations: CpG ODN, CpG oligodeoxynucleotide; HBsAg, Hepatitis B surface antigen; IL-12, interleukin-12; APC, antigen presenting cell; DC, dendritic cell.

Animals
Female C57BL/6, adult (6-8 weeks old) and aged (over 15 month), were mice purchased from the Research Center of Tissue Transplantation and Immunology of Jinan University.

Synthetic CpG ODN
Nuclease-resistant phosphorothioate CpG oligomers were synthesized by Shanghai Shenggong Biologic Engineering Company. The sequence of the ODN utilized in this study was designed as previously described: CpG 1826: 5'-TCC-ATG- ACG-TTC-CTG-ACG-TT-3' (12).

Preparation of the mixture of ODN and vaccine
330μg CpG ODN was dissolved in 1.65 ml sterile aqueous solution and mixed with HBsAg. The mixture was stored at 0°C before injection.

Immunization of mice
All aged mice were divided into 3 groups (n = 8). In the aged control group, mice were immunized with 100 μl HBsAg solution into the left tibialis anterior muscle. In the 10 μg CpG group, mice were immunized with the mixture of 10 μg CpG ODN and 100 μl HBsAg solution. In the 20 μg CpG group, mice were immunized with the mixture of 20 μg CpG ODN and 100 μl HBsAg solution. Adult control mice (n = 8) were immunized with 100 μl HBsAg solution. All mice were boosted after 2 weeks using the same formulation. Serum was collected 5 weeks after last immunization and stored at -20°C until antibody and cytokine analysis were performed.

Measurement of anti-HBs antibody, IL-12 and IFN-γ
Briefly, purified HBsAg, anti-IgG1, anti-IgG2a, anti-IL-12 and anti-IFN-γ antibody were diluted in phosphate buffered saline (PBS) and dispensed into 96-well culture plates, respectively. Following overnight of incubation at 4°C, the plates were washed extensively with PBS/0.05% Tween 20 and blocked with 10% bovine serum albumin (BSA) for 2h at 37°C. Serum samples, serial dilutions of standard IgG, IL-12 or IFN-γ were added to the plates and incubated 2h at 37°C. After extensive washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was diluted 1:1500 in PBS and sequentially added to the plates for 2h at 37℃. The plates were washed 4 times with PBS/0.05% Tween 20 and developed by adding the appropriate amounts of 2,2-azinobis (3-ethylbenzthioline-6-sulfonic acid) substrate and H_2O_2. After color development, the optical density readings were recorded at 490 nm. Standard reference curve was used to establish the amount of specific antibody contained in each unknown sample.

Pathological changes of spleen
To evaluate pathological changes of spleen lymphocytes, hematoxylin & eosin staining was performed. Briefly, all spleens were fixed with acetone for 10 minutes, and then they were dehydrated and immersed in paraffin. Immersed spleens were cut into paraffin slices, and the paraffin slices were dewaxed into water and stained by hematoxylin and eosin. Finally, they were dehydrated and enveloped. In optical microscope, pathological changes of spleen lymphocytes were observed.

Statistical analysis
Statistical analysis was determined by Student's paired *t*-test using SPSS software and each value was given as mean ± SD. Statistical significance was defined as $p<0.05$.

Results

Effect of CpG ODN on the production of anti-HBs antibody in aged mice
As shown in Table 1, compared with low levels of IgG2a, IgG1 was the predominant isotype in adult mice after last

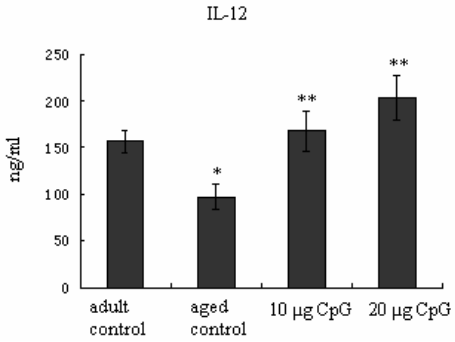

Figure 1. Effect of CpG ODN on the level of IL-12 in aged mice. Aged control group, 100 μl HBsAg; 10 μg CpG group, 10 μg CpG ODN and 100 μl HBsAg; 20 μg CpG group, 20 μg CpG ODN and 100 μl HBsAg. Adult control group, 100μl HBsAg. The level of IL-12 was detected by ELISA. *$p<0.05$ *vs* the adult control, **$p<0.001$ *vs* the aged control. Figure 1 showed CpG ODN could improve the cellular immunity against hepatitis B vaccine in aged mice and also showed the effect of CpG ODN was dose-depedent.

Table1. *Effect of CpG ODN on the production of anti-HBs antibody in aged mice.*

Group	Anti-HBs antibody (mg/L)			
	Total IgG	IgG1	IgG2a	IgG1:IgG2a
Adult control	57.20 ± 1.21	45.40 ± 1.82	4.13 ± 0.36	11:1
Aged control	38.90 ± 0.52*	36.38 ± 0.47*	1.07 ± 0.21*	34:1
10μg CpG	101.36 ± 1.87*	76.80 ± 1.56*	12.80 ± 1.20*	6:1
20μg CpG	162.70 ± 3.45*	103.00 ± 2.56*	20.60 ± 1.38*	5:1

*$p<0.05$. Serum anti-HBs antibody was determined by ELISA. All samples were determined within 3 months after collection.

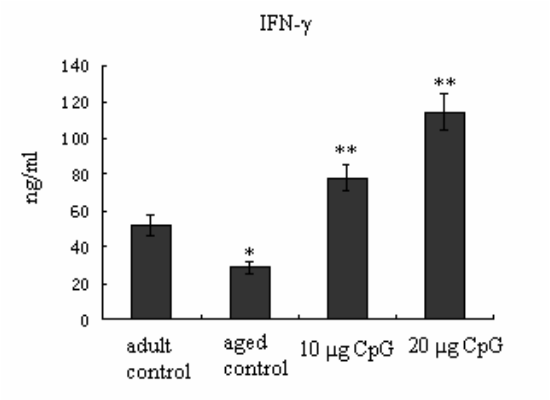

Figure 2. Dose-dependent effect of CpG ODN on the production of IFN-γ in aged mice. Adult control group, 100 μl HBsAg; Aged control group, 100 μl HBsAg; 10 μg CpG group, 10 μg CpG ODN and 100 μl HBsAg; 20 μg CpG group, 20 μg CpG ODN and 100 μl HBsAg. The concentration of IFN-γ was detected by ELISA. *$p<0.05$ *vs* the adult control, **$p<0.001$ *vs* the aged control. The figure showed CpG ODN could improve Th1 type response in aged mice and the effect was dose-dependent.

immunization with HBsAg alone, resulting in the IgG1:IgG2a ratio of 11:1. The level of IgG2a antibody produced by aged mice immunized with only HBsAg was lower than that of adult mice, which gave the IgG1:IgG2a ratio of 34:1. However, a significant increase in the levels of IgG2a was observed in the aged mice vaccined with HBsAg plus 10 μg CpG and 20 μg CpG. The combination of 10 μg CpG or 20 μg CpG with HBsAg vaccine can lower the IgG1:IgG2a isotype ratios to 6:1 and 5:1 respectively, indicating an increase in Th1 type response. In addition, total anti-HBs IgG in CpG-added groups can be seen to significantly increase.

Effect of CpG ODN on the production of Th1 type cytokines *in aged mice*

In order to understand whether the mixture of CpG and HBsAg vaccine could enhance Th1 type response, two Th1 type cytokines, IL-12 and IFN-γ, were detected by ELISA. As shown in Figure 1 and 2, the respective concentrations of IL-12 and IFN-γ were 97.80 ± 13.41 ng/ml and 28.91 ± 3.34 ng/ml in aged mice immunized with only HBsAg. They were significantly lower than the concentrations of IL-12 (156.26 ± 11.30 ng/ml) and IFN-γ (52.20 ± 5.71 ng/ml) in adult mice, suggesting Th1 type response in aged mice was low. The respective levels of IL-12 and IFN-γ significantly rose to 167.89 ± 22.14 ng/ml and 78.11 ± 7.32 ng/ml in aged mice injected with mixture of 10 μg CpG and similar HBsAg vaccine and were higher than those in aged control group. Moreover, the values of two cytokines in 20 μg CpG group were higher than those in 10 μg CpG group. These results showed CpG ODN could improve Th1 type response in aged mice and the effect was dose-dependent.

Proliferation of spleen lymphocytes in aged mice

In optical microscope, the pathological changes of spleen lymphocytes could be seen in Figure 3A~D. The number of lymphocytes in aged control group (Figure 3B) was less than that in adult control (Figure 3A). The number of lymphocytes in 10 μg CpG group was significantly more than that in aged control group and nuclei of lymphocytes became larger (Figure 3C). The proliferation of spleen lymphocytes in 20 μg CpG group was more significant than that in 10 μg CpG group (Figure 3D). These results showed that CpG ODN could stimulate proliferation of spleen lymphocytes in aged mice.

Discussion

There are numerous reports proposing that the declines in immune function with aging are due to the functional and phenotypic alterations that occur in T and B cells (3, 13-16). Moreover, the well-documented deficiencies in aged immune system may also involve depressions in antigen

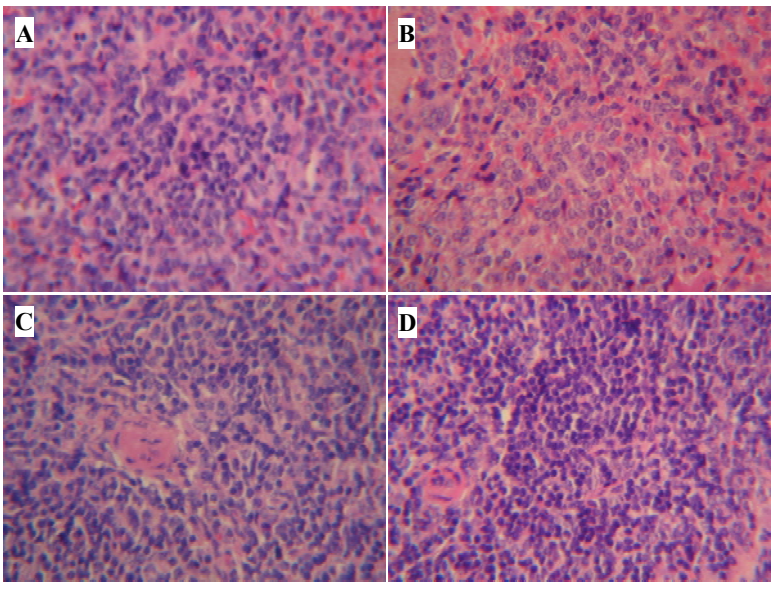

Figure 3. Proliferation of spleen lymphocytes in response to CpG ODN in aged mice. Hematoxylin & eosin staining was used to evaluate pathological changes of spleen lymphocytes (A) adult control group, 100 μl HBsAg. (B) aged control group, 100 μl HBsAg; (C) 10 μg CpG group, 10 μg CpG ODN and 100 μl HBsAg; (D) 20 μg CpG group, 20 μg CpG ODN and 100 μl HBsAg. The figure showed CpG ODN could stimulate proliferation of spleen lymphocytes in aged mice.

presenting cells (APCs) function. Dendritic cells (DCs) are professional APCs that can take up and present native antigen to B cells, or process antigen peptides to naive T cells via MHC I or MHC II molecules, thereby forming a link between the innate and acquired immune systems (17). A number of well-documented differences exist between young and old animals that could have inhibitory or stimulatory effects on the activation and maturation of DCs. For example, IL-10 and plasma glucocorticoid levels are elevated in aged animals (18). Factors such as TNF-α, prostaglandins and reactive oxygen species are produced at higher levels in aged individuals. These factors could lead to the premature activation and maturation of DCs prior to their encountering foreign antigens (19). Finally, IL-6, also known to be elevated in aged animals, has recently been showed to alter the DC processing and presentation of protein antigens, and to enhance the differentiation of committed precursor cells towards macrophages but not DCs (20). The deficiency in aged immune system makes aged individuals not only has a reduced capacity to be successfully vaccinated but also be short of protective immune responses against infectious agents. So it is important for aged individuals to improve their immunity to defend the infection of bacterial toxins. However, the aluminum-based mineral salts that have gained wide acceptance for uses in human vaccines are demonstrated to be minimally effective in elderly. Recently, prokaryotic DNA has been described as having potent stimulatory effects on a variety of immune cell types. Although the mechanisms responsible for the immunostimulatory effects are still unclear, extensive studies employing synthesized CpG ODNs have established a requirement for the presence of unmethylated CpG motifs. Numerous studies have showed that CpG ODN expressing CpG motifs activate an innate immune response characterized by the production of IgM, IL-6, IL-12, IL-18, TNF-α, and IFN-γ (21-27). Further experiments have demonstrated that CpG ODNs are able to directly influence B cells, macrophages and DCs following cellular uptake through absorptive endocytosis (28, 29) and/or following specific interactions with Toll-like receptors (30). CpG ODNs have also been shown to indirectly stimulate T cells and NK cells through their ability to promote secretion of IL-12 by macrophages and DCs (31, 32). In many cases, the immunostimulatory activity of CpG ODN relies on their ability to induce the production of IL-12 (33-36). Our studies investigated effect of CpG ODN on anti-HBs antibody, IL-12 and IFN-γ against hepatitis B vaccine in aged mice.

We used aged C57BL/6 mice and CpG 1826 that was optimal for mice as adjuvant, vaccinated with HBsAg. We found there were significant increases in total IgG and IgG1 and IgG2a when 10 μg and 20 μg CpG ODNs were mixed with hepatitis B vaccine in aged mice. There were significant increases in IL-12 and IFN-γ when CpG ODNs were mixed with hepatitis B vaccine in aged mice and the increase of 20 μg CpG group was more significant than that of 10 μg CpG group. In optical microscope, there were less lymphocytes in aged mice than those in adult mice. The proliferation of spleen lymphocytes in 20 μg CpG group was more significant than that in 10μg CpG group in aged mice. These results also showed that the enhanced response to HBsAg vaccine combined with CpG ODN was dose-dependent. Thus, our results demonstrated a utility for CpG ODN, as a safe vaccine adjuvant for promoting effective immune responses in aged mice. Recently, Halperin et al. showed that CpG added to HBsAg was well tolerated and immunogenic in phase I study in healthy adults and might offer the potential for enhancement of hepatitis B virus (HBV) immunization and protection after one or two doses or in individuals who failed to respond to the standard vaccine regimen (37). These agents could have important clinical uses in overcoming some of the age-associated depressions in immune function that occur in response to vaccination.

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